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A Novel Motif in HIV-1 Nef that Regulates MIP-1β Chemokine Release in Macrophages: A Dissertation

Lue Dai

University of Massachusetts Medical School

A Novel Motif in HIV-1 Nef that Regulates MIP-1β Chemokine Release in Macrophages

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Lue Dai

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A NOVEL MOTIF IN HIV-1 NEF THAT REGULATES MIP-1β CHEMOKINE RELEASE IN MACROPHAGES

A Dissertation Presented

By

LUE DAI

The signatures of the Dissertation Defense Committee signifies completion and approval as to style and content of the Dissertation

_____

Mario Stevenson, Thesis Advisor

_____

Paul Clapham, Member of Committee

_____

Heinrich Gottlinger, Member of Committee

_____

Mohan Somasundaran, Member of Committee

_____

David Evans, Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

_____

Maria Zapp, Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school.

_____

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

Program in Immunology and Virology
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The following figures are used with the permission of the collaborators:

Dr. Jean-Marc Jacque, Figure 3-1: Nef induces MIP-1β production independently of its other classical functions.

Dr. Simon Swingler, Figure 4-3: The KEK motif is required for Nef-dependent ferritin release from macrophages.

Dr. Ruzena Stranska, Figure 4-4: Induction of neurotoxicity in macrophage supernatants by Nef (lower two panels).

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Last but not least, I dedicate this thesis to my family, my parents Huping Dai and Peilan Xu, my wife Zhengxian Sheng, my baby boy Lucas Dai and my parents-in-law Jialong Sheng and Huizhi Xin, for their unconditioned love and unwavering support.

ABSTRACT

Nef is an accessory protein encoded by human and simian immunodeficiency viruses (HIV and SIV), and is critical for viral pathogenicity *in vivo*. The structure of Nef has been resolved and the major cellular activities of Nef are generally described as down-regulation of cell surface molecules, enhancement of virus infectivity and regulation of cell signaling and activation. Macrophages represent a key target of HIV-1 infection and may contribute significantly to viral pathogenesis by facilitating viral propagation, maintaining a viral reservoir and regulating viral replication. During HIV-1 infection, various cytokines and chemokines are induced for viral advantages more than for host defense.

We have previously demonstrated that HIV-1 Nef regulates the release of chemokines, MIP-1α and MIP-1β, from infected macrophages and have proposed that this may enhance conditions for viral replication by promoting recruitment of substrate lymphocytes to sites of infection (1). However, the molecular basis for this Nef activity remains to be defined. The main goals of this thesis are to identify the functional motif in Nef that is responsible for chemokine induction in macrophages and to elucidate the relevance of this motif to other Nef functions. Using a mutagenesis approach, we have eventually identified a novel motif (KEK) that regulates chemokine production in infected macrophages after we excluded several previously described Nef motifs. This motif is conserved in both HIV-1 and SIV Nef proteins. Mutations in this domain abrogated MIP-1β induction as well as the Nef-dependent release of other secretory

factors by macrophages. However, disruption of this motif did not affect other Nef-ascribed activities such as CD4 and MHC-I down-regulation. In addition, we have determined the involvement of viral Env proteins in Nef-induced chemokine production. Distinct signaling pathways that regulate chemokine release in macrophage will also be described. Finally, several possible roles of the KEK motif are proposed and some preliminary results of co-immunoprecipitation experiments will be presented which aim to characterize cellular proteins involved in chemokine regulation by Nef. Collectively, our studies reveal a specific determinant within Nef that is critical for chemokine release by Nef. Identification of this motif paves the way for future studies to explore the molecular machanisms of Nef-regulated cell signaling pathways. Such knowledge may point to new therapeutic strategies that interrupt Nef function and limit the course of HIV-1 infection.

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ABBREVIATIONS

aa	amino acid
AP	adaptor protein
APC	antigen presenting cell
ARF6	ADP-ribosylation factor 6
CA	capsid
CNS	central nervous system
CTD	C-terminal domain
CypA	cyclophilin A
Dyn2	Dynamin 2
Env	envelope
HAART	highly active antiretroviral therapy
HIV	human immunodeficiency virus
HTLV-III	human T-cell leukemia virus III
IN	integrase
IKK	IκB kinase
LAV	lymphadenopathy-associated virus
LTNP	long-term non-progressors
LTR	long terminal repeat
MA	matrix
MAPK	mitogen-activated protein kinase
MHC-I	major histocompatibility complex class I
NAK	Nef-associating kinase

NC	nucleocapsid
Nef	negative factor
ORF	open reading frame
PACS	phosphofurin acidic cluster sorting
PBL	peripheral blood lymphocyte
PI3K	phosoinositide 3 kinase
PIC	pre-integration complex
PR	protease
rNef	recombinant Nef
RRE	Rev-responsive element
RT	reverse transcriptase
SFK	Src-family tyrosine kinase
SIV	simian immunodeficiency virus
STLV-III	simian T-cell leukemia virus III
SU	surface unit
TAR	transactivation response
TGN	trans-golgi network
TM	transmembrane domain
Vif	viral infectivity factor
Vpr	viral protein r
Vpu	viral protein u
ZAP70	zeta chain associated protein 70kDa

CHAPTER I.

INTRODUCTION

1. HIV-1 and SIV

1.1. Primate lentivirus and AIDS

The lentivirus Human immunodeficiency virus type 1 (HIV-1) was first identified in 1983 when Luc Montagnier's group at the Pasteur Institute isolated a novel retrovirus named lymphadenopathy-associated virus (LAV) (2). Robert Gallo's group at NIH also identified the virus and named it as human T-cell leukemia virus III (HTLV-III). They confirmed that this was the pathogen that caused acquired immunodeficiency syndrome (AIDS) in humans (3). Two years later, simian T-cell leukemia virus III (STLV-III), now referred to as simian immunodeficiency virus (SIV), was isolated from rhesus macaques with an AIDS-like illness and this virus presented similar morphology, growth pattern and antigenic properties as HTLV-III, providing the first evidence of the close relationship between SIV and HIV (4) . It is now widely believed that SIV from African primates may have crossed species from monkeys to humans resulting in different HIV strains. HIV-1 may have originated from the evolution of SIVcpz, an SIV strain from infected chimpanzees (5, 6), while the less pathogenic HIV-2 appears to be a relative of SIVsm, an SIV strain from infected sooty mangabeys (7),(8).

HIVand its relative SIV are primate lentiviruses belonging to the *retroviridae* family. The "retro-" in the family name emphasizes a unique step in replication of these RNA viruses: synthesis of viral DNA from an RNA genome by reverse transcriptase, while the "lenti-" in the genus name stands for a slow incubation time usually followed by a latent

but eventually destructive infection. Since the first five AIDS patients were reported in 1981, HIV infection has killed approximately 30 million people. According to a 2009 UNAIDS report, about 33.4 million people are living with HIV infection. Most untreated HIV-1 infected people will develop AIDS and die from opportunistic infections or tumors due to the total failure of the immune system. Unfortunately, there is currently no cure for HIV infection, although a cocktail therapy with combined usage of at least three antiretroviral drugs, referred to as highly active antiretroviral therapy (HAART), has successfully controlled HIV infection and reduced AIDS mortality by suppressing viral replication. This therapy, however, has to be continuous since it is unable to completely clear a latent viral reservoir from the host. This makes the treatment expensive. For this reason, the majority of HIV infected people in developing countries are still short of proper treatment and medication. An affordable vaccine, considered to be the ultimate solution, has not been developed. In September 2009, a combination of two previously failed vaccine candidates was reported to reduce HIV infection by 30% according to a vaccine trail in Thailand (9). The news is encouraging but it is still far from a preventive vaccine, especially considering the numbers of the infected people in the vaccine and the placebo groups were actually very close (51 vs.74) making the significance of the results questionable. In addition to the development of novel drugs and vaccines, restoration of the impaired immune system of HIV patients using bone marrow or stem cell transplant may offer another option to finally cure AIDS in the future (10).

1.2. HIV-1 genome and viral proteins

The HIV-1 genome, depending on the stage of the viral replication cycle, may be present in RNA or DNA form. In a typical viral particle, the viral genome appears as two copies of single-stranded RNAs whereas in an intracellular pre-integration complex (PIC) it becomes double-stranded DNA after reverse transcription. On both ends of the genome are two identical sequences termed as 5'- and 3'- long terminal repeats (LTRs) and between them there are 9 open reading frames (ORF) (Fig. 1-1). The *gag*, *pol* and *env* are the three fundamental genes common to all retroviruses, and in HIV-1 they initially encode polyprotein precursors that are processed into individual viral proteins. According to their distinct functions, the viral proteins of HIV-1 can be categorized into four groups: the structural proteins encoded by *gag* and *env*, the enzymatic proteins encoded by *pol*, the regulatory proteins Tat and Rev, and the accessory proteins Vif, Vpu, Vpr and Nef. These viral proteins exhibit a multitude of activities that can act in concert and contribute to the pathogenicity of HIV-1. The functions of each viral protein have been reviewed in detail (11).

The *gag* gene encodes a polyprotein precursor p55 that is cleaved by viral protease into matrix (MA/p17), capsid (CA/p24), nucleocapsid (NC/p7) and P6, along with two additional spacer peptides, p1 and p2. The MA domain of the p55 directs Gag to the cellular membrane after its synthesis (12) and also facilitates the incorporation of the Env proteins into virions (13). The structural function of CA protein is to form the viral core (14) but it also affects viral infectivity, probably through its association with

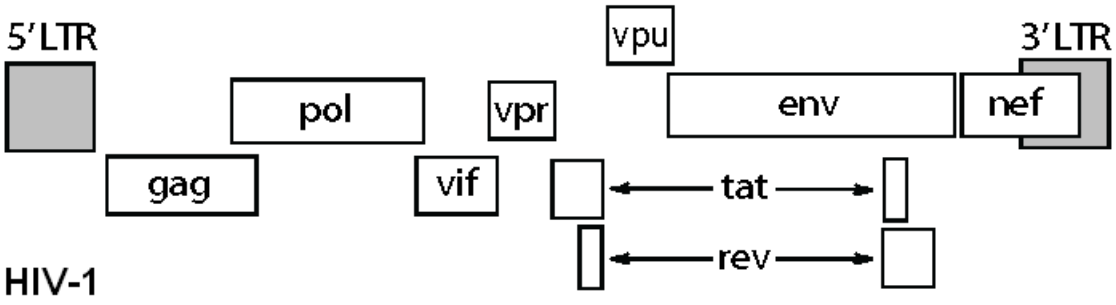

Figure 1-1: Genomic organization of HIV-1. LTRs are shown as grey boxes and ORFs as blank boxes.

cyclophilin A (CypA) during the viral uncoating step (15, 16). NC specifically binds to the packaging signal (Ψ-site) of the unspliced genomic RNA and directs them into virions (17). P6 promotes virus release by interacting with Tsg101 (18, 19) and P6 also facilitates the incorporation of the accessory protein Vpr into virus particles (20) .

The *env* gene encodes viral envelope (Env) as a glycoprotein precursor gp160 that is cleaved by a cellular protease into the surface unit (SU) gp120 and the transmembrane domain (TM) gp41. The spike unit gp120 interacts with its receptor CD4 and co-receptors CXCR4 or CCR5 on target cells and thus determines viral tropism. The transmembrane unit gp41 binds to gp120, stabilizes the gp120/gp41 complex and mediates fusion between viral and host lipid membranes during virus entry (21).

A frameshift event during Gag translation leads to the synthesis of three *pol* gene products, protease (PR), reverse transcriptase (RT) and integrase (IN), as part of a large Gag-Pol precursor. The first substrate of PR is likely to be itself so that it can be released and continue with the subsequent processing of the Gag and Gag-Pol precursors, which is required for virion maturation. RT generates the double-stranded DNA copy from the single-stranded RNA viral genome in infected cells. It has both RNA- and DNA-dependent polymerase activities and its RNase H domain removes the RNA template from the RNA/DNA hybrid during double-stranded DNA synthesis. As an error prone polymerase, RT causes a high mutation rate during viral replication (22), which is an efficient way for HIV-1 to evade antibodies and antiviral drugs. After reverse transcription, IN catalyzes the integration of viral DNA into the host genome. This

includes at least 3 steps, 3' processing of the viral DNA, strand transfer and gap repair, which lead to the formation of the integrated provirus.

Tat and Rev are two regulatory proteins that enhance viral gene expression. Tat interacts with the transactivation response (TAR) element in the 5'-LTR and functions to stimulate the LTR-directed transcriptional elongation by recruiting a cyclin T1/CDK9 complex to hypophosphorylate the C-terminal domain (CTD) of RNA polymerase II (23, 24). Rev serves as a nuclear exporter for unspliced genomic viral RNA or partially spliced viral mRNAs by binding to the Rev-responsive element (RRE) (25, 26). Tat and Rev are indispensable for a complete viral replication cycle because the efficient expression of most viral proteins is largely dependent on them.

The HIV-1 genome encodes an additional four proteins, viral infectivity factor (Vif), viral protein u (Vpu), viral protein r (Vpr) and negative factor (Nef). These are collectively termed "accessory" proteins due to the fact that their absence usually has little effect on viral replication in cell cultures. However, recent studies have revealed that they carry important activities that support viral replication. Vif enhances virus infectivity by countering the antiviral effect of APOBEC3G (27). Vpu is reported to overcome another cellular restriction factor tetherin/BST-2 that prevents virus release (28) and it also down-regulates intracellular CD4 expression (29), which helps to avoid envelope interference. Vpr enhances viral replication in nondividing macrophages (30) possibly by facilitating nuclear import of the HIV-1 PIC (31, 32) and it is also known to induce cell cycle arrest in G2 phase (33, 34). My thesis research is mainly focused on the accessory protein Nef,

which is probably the most multifunctional viral protein of HIV-1. Studies dealing with Nef structure and activities will be reviewed in detail in the Chapter I-2.

1.3. HIV-1 replication cycle

Productive HIV-1 infection requires that virions go through a complete HIV-1 replication cycle which includes the following critical steps: receptor/co-receptor binding, membrane fusion, virion uncoating, reverse transcription, PIC nuclear import, viral DNA integration, viral mRNA production and nuclear export, viral protein translation, virion assembly, budding and maturation (reviewed in Fields Virology (11), Figure 1-2).

The viral entry step is initiated when gp120 binds to the CD4 receptor on the cell surface of the target cells. CD4 binding alone, however, is not sufficient for virus entry. A subsequent conformational change in gp120 promotes its additional engagement with co-receptor molecules including CCR5 and CXCR4 on macrophages and T cells, respectively. Viruses using CCR5 replicate well in macrophages and are termed R5- or M-tropic viruses while viruses using CXCR4 mainly infect T cells and are termed X4- or T-tropic viruses. In addition, there are some dual tropic HIV-1 isolates that can infect both cell types (35, 36). After gp41-mediated lipid fusion of the viral and cellular membranes, the uncoating process exposes the PIC in which MA, RT, IN and Vpr remain associated with the viral RNA genome. RT then completes the reverse transcription step to synthesize a cDNA copy of the viral genome which is transported into the nucleus as part of the PIC. In the nucleus, IN inserts the viral cDNA into the host chromosome, making HIV-1 infection a permanent genetic event in the infected host cells. Using the integrated proviral DNAs as templates, host RNA polymerases initiate the LTR-directed transcription to generate the viral mRNAs, which is markedly enhanced by Tat.

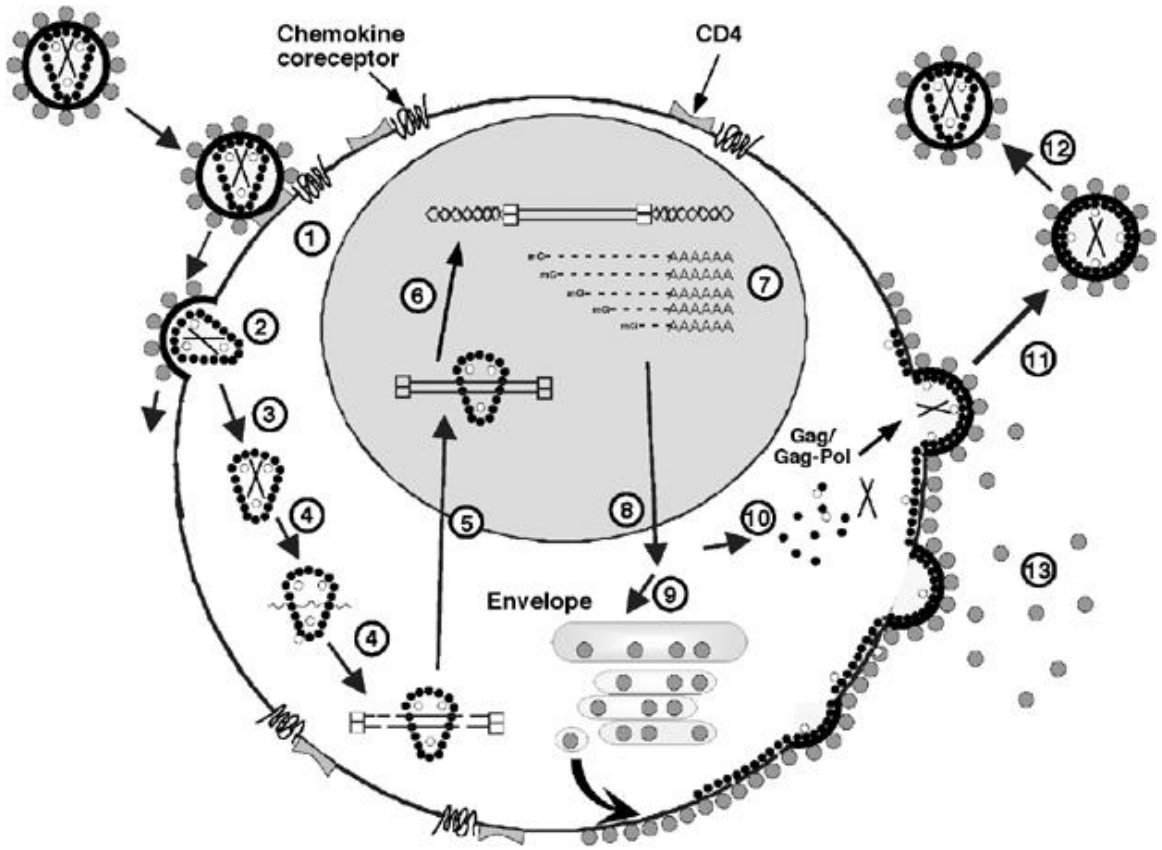

Figure 1-2: HIV-1 replication cycle. The critical replication steps include: (1) receptor/co-receptor binding; (2) membrane fusion; (3) virion uncoating; (4) reverse transcription; (5) PIC nuclear import; (6) viral DNA integration; (7-8) viral mRNA production and nuclear export; (9-10) viral protein translation and virion assembly; (11) virion budding and (12) virion maturation. (This figure is adapted from Fields Virology (11)).

Unspliced or partially spliced viral mRNAs with the RRE sequence are recognized and transported by Rev through the cellular nuclear export machinery into cytoplasm where the translation of viral proteins occurs. The Env, Gag and Gag-Pol polyprotein precursors then gather at plasma membrane for the virus assembly and budding. NC recognizes the ψ –site on the viral genomic RNA and has it packaged into the newly assembled virions. During the virus release, PR cleaves the Gag and Gag-Pol precursors for the final virion maturation.

2. Nef

2.1. Nef and viral pathogenesis

Nef is one of the accessory proteins that are encoded only by HIV and SIV. In the HIV-1 genome, the *nef* gene is positioned following the 3'-end of the *env* gene with most of its 3'-half overlapping with the 3'-LTR (Fig. 1-1) and encodes a 27kDa protein. In SIV or HIV-2, the *nef* gene begins before the *env* gene ends, thus encoding longer sequences at the N-terminus of the gene products. Nef is one of the first synthesized viral proteins, it is highly expressed in infected cells and it is myristoylated at the N-terminus. While an approximate 30% proportion of Nef is membrane-asscoiated, Nef is found predominantly in the cytosol (37-39). Nef is also found to be incorporated into virions where it can be further processed by the viral protease (40-42).

Although the name "Nef" stands for "Negative factor", because some early studies described it as a gene suppressor for LTR-driven transcription (43, 44), it is now widely agreed that Nef plays a critical role in viral pathogenesis and disease progression *in vivo*, making Nef a very attractive drug target. A complication of Nef research, however, comes from multiplicity of various Nef functions discovered in distinct *in vitro* systems. Thus, one important goal of Nef research is to understand the independent contribution of each Nef activity to the totality of Nef function and to viral pathogenesis so that effective antiviral drugs targeting specific Nef activities can be developed.

Although AIDS is usually a rapidly progressive disease, less than five percent of HIV-1 infected patients are reported as long-term non-progressors (LTNPs). These patients remain infected but do not succumb to AIDS even without antiviral treatment and they usually have very low viral titers and reasonably stable CD4+ T cell counts (45, 46). The most extensively studied LTNP group is the Sydney Blood Bank Cohort that comprises six blood product recipients who got infected with HIV-1 from a single donor (47). They remained free of AIDS after more than ten years of infection and it was later discovered that their virus isolates had a similar deletion in the *nef* gene. Analysis of the molecular nature of the virus isolates from several other LTNPs also revealed defects in *nef* genes (48-50). A study that compared the *nef* genes of virus isolates from AIDS and LTNP patients has correlated some common mutated Nef residues to AIDS progression (51).

The possibility that the absence of an intact *nef* gene could be responsible for the LTNP phenotype is supported by observations in SIV-infected monkeys. Adult rhesus macaques infected with cloned SIVmac239 viruses whose *nef* gene was deleted were persistently infected with low viral loads but did not progress to AIDS (52, 53). The premature stop codon introduced in the *nef* gene of SIVmac239 reverted back to normal very rapidly post infection (52). Other primate infection studies using SIV variants harboring a 12-bp *nef* deletion (54) or other *nef* gene mutations (55, 56) reveal a similar selective pressure for an intact and functional *nef* to accelerate viral replication and disease progression *in vivo*.

Construction of transgenic mice that express Nef alone in T cells offers a valuable model to understand the relationship between Nef and pathogensis *in vivo*. Nef expression

exclusively in T cells was achieved by putting the *nef* transgenes under the control of the regulatory elements of CD2, CD3, TCR and CD4 genes (57-60). Transgenic Nef expression led to abnormal T cell development and activation, as well as subsequent T cell depletion. Furthermore, a severe AIDS-like disease was observed in a mouse model where transgenic Nef expression was limited to CD4+ T cells and cells of the monocyte/macrophage lineage (60).

Collectively, *in vivo* studies in humans, non-human primates and transgenic mice all indicate Nef as a critical determinant for viral pathogenicity and disease progression.

2.2. Structure of Nef

The structure of the 27kDa HIV-1 Nef protein can be described in two separate parts: a highly variable N-terminal membrane anchoring domain and a highly ordered C-terminal core domain that is well conserved among HIV-1 Nef alleles. It has been shown that PR is able to separates these two domains at a specific cleavage site $W^{57}L$ (note: all residue numbers in this thesis are according to the sequence of HIV-1 NL4.3 Nef) (61, 62), which may explain the release of Nef core domain in mature virions. The structure of HIV-1 Nef domains has been defined in NMR solution (63, 64) as well as in crystal form (65, 66) (Fig. 1-3). Although very little structural work has been done for SIV or HIV-2 Nef, similar core domain structure can be predicted by the high similarity of the residues in their core domains.

The first 6 amino acids (M^1GxxxS/T) constitute the most important domain on the N-terminal arm of HIV-1 Nef: the myristoylation sequence (67). The G^2 residue is the myristoylation site where a myristate is covalently linked by a cellular N-myristyl transferase. The N-terminal myristyl modification is required for Nef to attach to the cell membrane and to perform most of its functions. The structure of the N-terminal domain of Nef has been described (64) (Fig. 1-2A). In the unmyristoylated form, the N-terminal domain of HIV-1 Nef is mostly unstructured with only one short alpha helix. Myristoylation, however, seems to stabilize the formation of a second alpha helix. Other than that, the N-terminal domain of Nef is very flexible and ready for conformational changes.

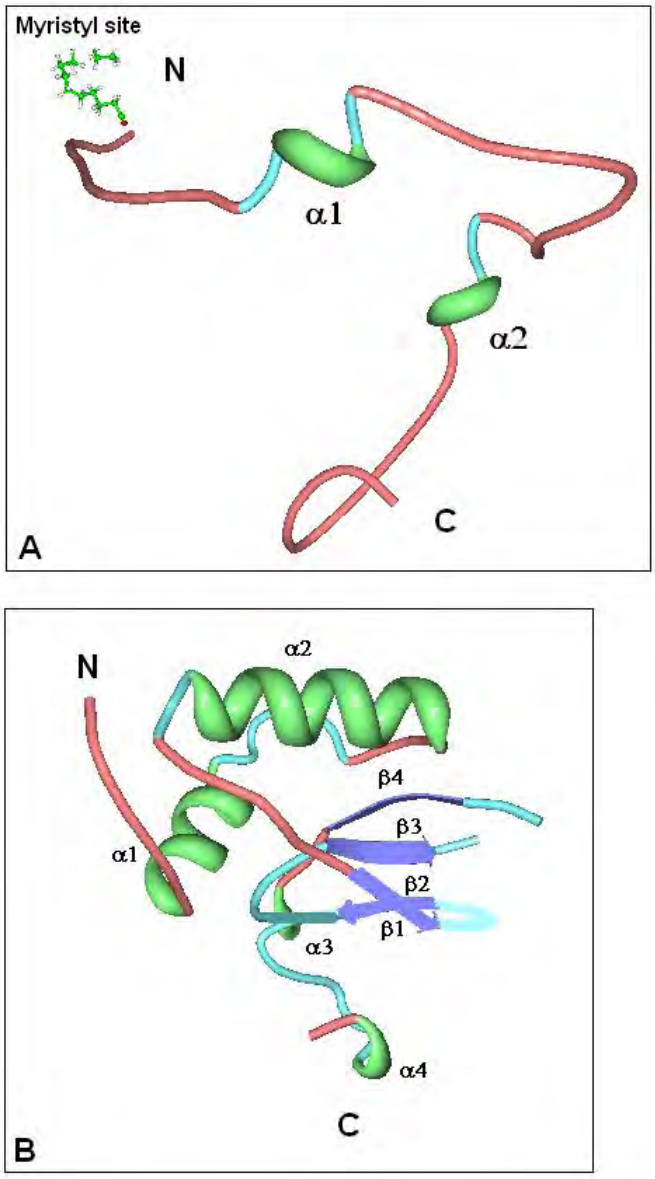

Figure 1-3: Structure of Nef domains. A. The structure of the N-terminal domain (aa 2-57) of Nef in NMR solution (pdb code 1Q45). B. The structure of the Nef_{core} (aa 71-202) in crystallization (pdb code 1AVV). Secondary structures are labeled and shown as green for α helices and cyan for β sheets.

The core domain of Nef (Nef_{core}) contains highly ordered structures that are important for interactions with different cellular proteins (Fig. 1-2B). Specifically, the Nef_{core} consists of one type II helix (aa 70-77), four alpha helices, α1, α2 (aa 81-120) and α3, α4 (aa 187-203), and a four-stranded beta sheet (aa 121-186). When the Nef_{core} was co-crystallized with the SH3 domain of Fyn, the polyproline type II helix of the Nef_{core} seemed to be at the interface, and based on this, the $P^{72}XXP$ motif of Nef has been known as the main SH3 binding site for Src family kinases such as Lck, Fyn and Hck (65, 66, 68, 69). Following this polyproline helix, the longer α1, α2 helices connected with a flexible loop form a cavity that is theoretically accessible to a potential binding partner. The two proline residues (P^{136} and P^{147}) twist the beta sheet structure and result in two pairs of anti-parallel strands that contribute to the hydrophobic groove of the Nef_{core}. In addition, there is another long flexible loop between β3 and β4, which is also open for potential protein interactions.

2.3. Cellular functions of Nef

Nef is probably the most multifunctional viral protein of HIV-1. Extensive studies have documented at least three major cellular activities of Nef *in vitro*: down-regulation of cell surface marker, enhancement of virus infectivity, and regulation of cell signaling and activation. These activities are mechanistically distinct and genetically separable, which means Nef requires specific motifs for specific functions (Fig. 1-4). These three major cellular activities of Nef are individually reviewed below.

2.3.1. Down-regulation of cell surface markers

1). CD4

The first characterized function of Nef is the down-modulation of CD4 molecules on the cell surface (70, 71). As one of the most important cell surface markers of helper T cells, CD4 plays an essential role specifically in T cell antigen recognition and subsequent activation by binding MHC type II molecules on antigen presenting cells (APCs). Thus, a stable population of CD4+ T cells must be maintained for a functional immune system. The clinical hallmark of immune system dysfunction caused by AIDS is the depletion of CD4+ T cells.

For viral replication, CD4 serves as the primary receptor on target cells to initiate viral entry. However, it is to be removed post entry by viral mechanisms since constant CD4 expression at a high level promotes super-infection, prevents virion release and interferes

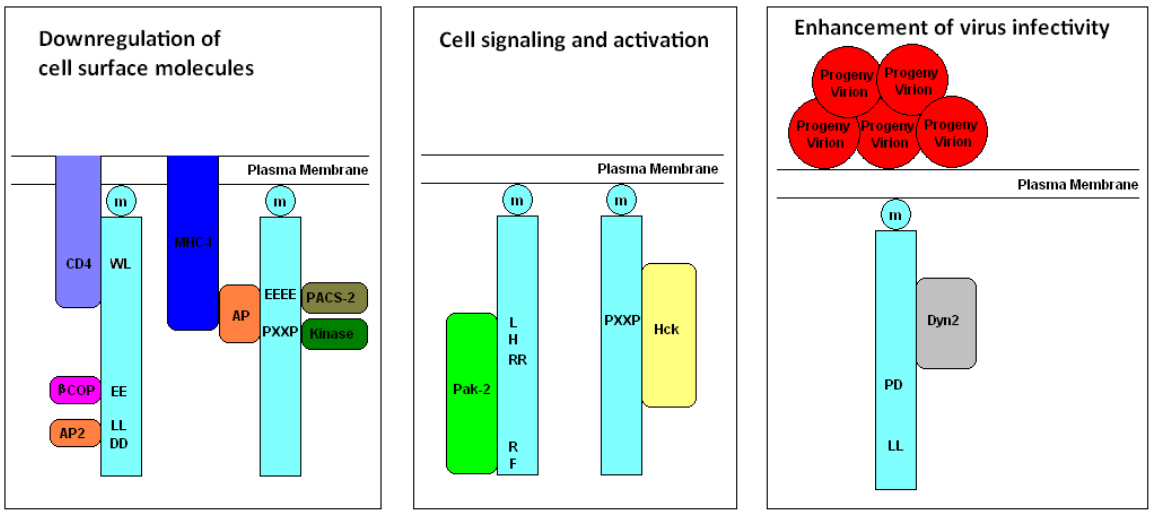

Figure 1-4: Cellular activities of Nef. HIV-1 Nef protein is shown in light blue and the critical residues for each function are indicated.

with viral envelope trafficking (72-75). Three viral proteins, Env, Vpu and Nef, have the ability to down-regulate CD4 through different mechanisms (75-78). Unlike Env and Vpu that mainly target the newly synthesized CD4, Nef is expressed at the early stage of the virus life cycle and dramatically down-regulates CD4 directly from the cell surface. The N-terminal myristoylation sequence of Nef is required for this function (79).

Nef has been shown to localize to clathrin-coated pits where CD4 molecules are enriched (80). It has been shown that the inhibition of clathrin-mediated endocytosis blocks CD4 down-regulation induced by Nef (81). These studies suggest a model in which Nef down-regulates CD4 by inducing clathrin-mediated internalization of these molecules from the cell surface and directing them to the endocytosis machinery for degradation. The internalization of CD4 by Nef involves direct binding of Nef to the cytoplasmic domain of CD4 (82), which has also been confirmed by the NMR analysis (83). The $W^{57}L$ motif of Nef seems to be at the interface of this interaction and is functionally required for CD4 down-regulation (84). After CD4 is internalized, Nef binds adaptor protein 2 (AP-2) that targets Nef-associated CD4 to lysosomes. The major motif of Nef for binding AP-2 has been mapped to the dileucine $L^{164}L$ in the flexible loop (aa 148-180) within the Nef_{core} (63, 85, 86). A second diacidic motif $D^{174}D$ within the same loop seems to enhance this interaction (87). Mutation of either the $L^{164}L$ or the $D^{174}D$ motif to alanines abrogates CD4 down-regulation by Nef. In addition, another motif responsible for CD4 down-regulation has been mapped to $E^{154}E$. This motif is required for the interaction of Nef with β-COP which directs CD4 from endosomes to lysosomes for degradation (88). Recently, ubiquitination of the K^{144} residue of Nef was found to be important for CD4

down-regulation, which indicated another pathway of Nef-mediated receptor degradation (89). Finally, CD4 down-regulation can also be regulated by the $P^{122}D$ motif of Nef by an unknown mechanism (90).

2). MHC-I

Major histocompatibility complex class I (MHC-I) represents another group of important cell surface markers down-regulated by Nef (91). Normally, MHC-I displays the peptides of foreign antigens, for example those of the viral proteins in the host cells, to CD8+ cytotoxic T (CTL) cells so that infected cells can be killed. This immune response is further enhanced by natural killer cells (NK) that can neutralize cells exhibiting an abnormal reduction of MHC-I expression. Nevertheless, Nef seems to keep the balance for HIV-1 infected cells to evade both CTL attack and NK surveillance by selectively removing particular groups of cell surface MHC-I molecules, HLA-A, B, but not HLA-C, E (92). The functions of Nef to down-regulate MHC-I and CD4 are mechanistically and genetically separate. Currently, there are at least two models of MHC-I down-regulation: one is focused on the phosphofurin acidic cluster sorting protein (PACS) and the other on the adaptor protein (AP).

An early study has suggested a PACS-dependent pathway of MHC-I down-regulation by demonstrating the interaction between Nef and PACS-1 (93). This interaction seems to override the ADP-ribosylation factor 6 (ARF6)-related endocytosis pathway in a phosoinositide 3 kinase (PI3K)–dependent manner, and as a result, MHC-I is directed to and retained in the trans-golgi network (TGN) (94). Recently, the PACS model has been

updated with studies on the Nef/PACS-2 complex and more details especially regarding the kinase cascade have been revealed (95, 96). It has been shown that once the Nef/PACS-2 complex arrives at the TGN, PACS-2 is detached while Src-family tyrosine kinase (SFK) binds Nef to phosphorylate a zeta chain associated protein 70kDa (ZAP70). ZAP70 then activates PI3K which leads to GTP loading of ARF6. This eventually accelerates the endocytosis of MHC-I molecules. Therefore, two Nef motifs appear to be critical for MHC-I down-regulation: the acidic $E^{62}EEE$ domain required for the PACS binding and the polyproline $P^{72}XXP$ domain important for SFK association.

The AP-dependent model is based on a ternary complex consisting of Nef, AP-1 and MHC-I (97, 98). In this model, Nef serves as a connector since AP-1 does not directly bind the cytoplasmic tail of MHC-1. This complex may direct newly synthesized MHC-I from the TGN to intracellular lysosomes for degradation. Interesting, formation of the complex is independent of the canonical AP binding motif $L^{164}L$; instead, it requires the same $E^{62}EEE$ and $P^{72}XXP$ motifs as in the PACS-dependent model of MHC-I down-regualation. Indeed, some data support that the $E^{62}EEE$ actually mediates the association of Nef with AP rather than with PACS (99). At present, whether and how these two models can be consolidated remains unknown.

3). CD3

Nef-dependent CD3 down-regulation is not conserved among Nef alleles. While most SIVs from old world monkeys such as SIVsm and their close relative HIV-2 encode Nefs with the ability to down-regulate CD3, this function appears to be lost in SIVcpz, and this

functional loss may have occured in HIV-1 (100). This raises an interesting explanation for the non-pathogenicity of most SIVs and HIV-2 in their natural hosts which is in contrast to the severe virulence of HIV-1 in humans. The hypothesis correlates the pathogenicity of lentiviruses with the ability of their Nef proteins to down-regulate CD3. By down-regulating CD3, SIVs and HIV-2 Nef proteins may help to prevent the hyperactivation of infected T cells and subsequent cell death. Since HIV-1 Nef fails to down-regulate CD3, HIV-1 infection in humans may proceed to AIDS more readily due to dramatic T cell loss and collapse of the immune system.

However, this model has been challenged due to the difficulty to explain some exceptions. The non-pathogenicity of SIV and HIV-2 is not absolute. When the species barrier is crossed, SIVsm can cause AIDS in rhesus macaques and black mangabeys, although their Nef proteins are still capable of down-regulating CD3 (101, 102). Even in natural hosts, there are exceptional cases of AIDS for SIVsm and HIV-2 infection with down-regulation of CD3 by functional Nefs (103-105). In the case of patients with dual infection of HIV-1 and HIV-2, CD3 down-regulation by HIV-2 Nef does not prevent AIDS progression caused by HIV-1 (106).

The significance and mechanism of CD3 down-regulation remains unclear in HIV-1 infection. Therefore, a priority is to identify the Nef domain that is responsible for CD3 down-regulation before the hypothesized rule can be further evaluated. Thorough determination of whether HIV-1 with a Nef mutant able to down-regulate CD3 loses its

pathogenicity, or alternatively, whether SIVs become virulent with a Nef mutant that fails to down-regulate CD3, needs to be performed.

4). Chemokine receptors

Chemokine receptors are important cell surface proteins normally found on leukocytes. They bind chemokines that induce leukocyte activation and migration. Active secretion of chemokines acts like a triggered alarm to the immune system, recruiting leukocytes into tissues where they are needed for a proper immune response. In the case of HIV-1 infection, two chemokine receptors CXCR4 and CCR5 serve as the co-receptors for viral entry and thus determine the tropism of HIV-1 infection. In the early stage of HIV-1 infection, viruses seem to prefer CCR5, whereas in the late stage of AIDS progression viruses tend to switch to use CXCR4 (107). Genetically, a 32bp deletion mutant of CCR5 (CCR5Δ32) has been reported to confer resistance to HIV-1 infection in an infected slow progressor (108), suggesting CCR5 as an attractive antiviral target.

As with CD4 down-regulation, Nef induces CXCR4 and CCR5 down-regulation to prevent superinfection (72, 109, 110). This activity is conserved among HIV-1, HIV-2 and SIV Nef proteins. CCR5 down-regulation is mediated by Nef probably through a similar endocytosis mechanism, since the signature motifs are mapped to $P^{72}XXP$ and $E^{62}EEE$ that are responsible for MHC-I down-regulation (111).

2.3.2 Enhancement of virus infectivity

Although Nef is not strictly required for viral replication in cell cultures, evidence from several groups has suggested that Nef functions to enhance infectivity of progeny virions (41, 112-115). This Nef function, however, appears to vary depending on the viral isolates. For example, HIV-1 SF2 Nef could increase viral infectivity by 38-fold while NL4.3 Nef only induced a 3-fold increase tested in the same assay (116). The Nef protein encoded by HIV-1 F12 strain even reduced virus infectivity, and interestingly, the inhibitory effect could be transferred to HIV-1 NL4.3 Nef with a single amino acid change E^{177}/G (117), suggesting Nef harbors determinants for virus infectivity. Like most HIV-1 Nef alleles, SIV Nef is also able to enhance virus infectivity and they are functionally interchangeable (118).

Expression of Nef in virus-producing cells rather than in target cells is believed to enhance infectivity of progeny virions (113-115). To be functional, Nef must be attached to the cell membrane and N-terminal myristoylation of Nef is required (41). Nef may enhance virus infectivity via specific attachment to lipid rafts (119, 120). However, this seems to be at odds with the result that MA, which controls the viral interaction with lipid, is not a target of Nef to boost infectivity (121), as well as the observation that a Nef mutant that is highly accumulated in lipid rafts fails to enhance infectivity (122). Contradictory results have been shown regarding whether Nef has to be packaged into virions to enhance infectivity. On one hand, blocking the binding of a fusion protein CypA-Nef to Gag prevents its incorporation into virions and inhibits its infectivity

enhancement effect (123). Yet in another experiment, virion-associated Nef did not enhance virus infectivity when it was packaged as a Nef-CS-Vpr fusion and was released into virions after processed by viral protease (124). Furthermore, most packaged Nef proteins are processed by PR into the Nef_{core} domain, but they are not sufficient to increase virus infectivity and the cleavage by PR is believed to be functionally irrelevant (125, 126).

Nef enhances the infectivity of HIV-1 viruses with both X4- and R5-tropic envelopes as well as viruses pseudotyped with amphotropic MLV envelope (127). However, similar effects were not observed with VSV-G- and Ebola-GP-pseudotyped viruses (128), which undergo a low-pH dependent endocytosis pathway for viral entry. A possible explanation is that Nef alters an actin cytoskeleton barrier in target cells that blocks post entry infection of ΔNef virus (129), and low pH-dependent envelopes may bypass the actin structure barrier by transporting ΔNef virus through the endocytosis machinery. Moreover, HIV-1 Nef is proposed to enhance virus infectivity, at least partly, by increasing the virion incorporation of viral envelope products (130).

The infectivity enhancement function of Nef can be uncoupled from other Nef functions *in vitro*. Although defective Nef mutants in terms of CD4 and MHC-I down-regulation as well as Pak2 association may still be able to enhance virus infectivity (126, 131, 132), mutations in the $L^{174}L$ motif responsible for AP-mediated CD4 down-regulation impaired the infectivity enhancement of Nef (133). In support of this, the infectivity enhancement by Nef was highly dependent on its cellular binding partner Dynamin 2 (Dyn2), which is

also needed for clathrin-dependent endocytosis induced by Nef. Presumably, Nef may bind Dyn2 to optimize virus infectiviy by down-regulating a potential restriction factor that targets Env in producer cells through the endocytosis pathway (134).

2.3.3. Regulation of cell signaling and activation

Efficient HIV-1 replication and spread is highly restricted in quiescent T cells as well as in undifferentiated monocytes. Proper activation and differentiation of these natural target cells of HIV-1 seems to enhance viral propagation. Nef is a possible factor to regulate cell activation by hijacking signaling pathways through its interaction with cellular kinases, among which Pak2 and Hck are the most extensively studied.

1). Pak2

Pak2 is a well-known Nef-associating kinase (NAK). Initial studies identified a cellular serine kinase that co-immunoprecipitated with Nef in human T lymphocytes (135). This serine kinase was later defined as a p21-associated kinase 2 (Pak2) that was activated by Rac and Cdc42 (136-138). Nef not only binds Pak2 but also has the ability to activate Pak2 (139), and this seems to be a common feature of HIV and SIV Nef proteins (139, 140).

Mutational analysis has determined several critical Nef domains for Pak2 association and activation. A specific determinant of Nef/Pak2 interaction has been mapped to L^{85}, H^{89}, R^{188} and F^{191}, which are proposed to form the hydrophobic binding surface for Pak2 (141). Substitution of these residues specifically prevents Pak2 association without

affecting other Nef functions such as CD4 or MHC-I down-regulation (141). Several other motifs are also indispensable. Mutating the myristoylation site residue G^2 severely impairs formation of the Nef/Pak2 complex suggesting the importance of membrane anchoring of Nef for Pak2 association (142, 143). The SH3 binding motif $P^{72}XXP$ is required for Pak2 activation possibly by the recruitment of Vav rather than by structurally participating in direct binding interaction (143-145). Moreover, mutation of two arginine residues $R^{105}R$ abrogates Pak2 association and activation (143), but the mechanism is yet to be determined. It should be noted that mutation of G^2, $P^{72}XXP$ or $R^{105}R$ also has some impacts on other Nef activities such as CD4 and MHC-I down-regulation.

The pathological significance of Nef/Pak2 interaction is still controversial. Nef-induced Pak2 activation may lead to T cell activation which stimulates viral replication (136, 146), or alternatively may trigger anti-apoptotic signaling pathways in infected T cells which might contribute to maintaining a virus reservoir (147, 148). However, a Pak2-binding deficient mutant $F^{191}R$ has hardly any effect on HIV-1 replication, T cell activation or apoptosis (149). Neverthless, Nef-induced Pak2 activation seems to be dependent on another Nef binding partner: a guanine nucleotide exchange factor Vav, and the interaction between Nef and Vav may lead to cytoskeletal rearrangements (145). If the actin barrier does exist for HIV-1 infection, the correlation of Pak2 activation with infectivity enhancement by Nef might be partly explained (143).

2). Hck

Nef has been reported to interact with several Src family tyrosine kinases (SFK). The interaction between Nef and the tyrosine kinase Hck is the best characterized. The structure of the Nef/Hck complex has been solved and the interaction involves the polyproline motif $P^{72}XXP$ of Nef and the SH3 domain of the kinase (63). Nef requires the same $P^{72}XXP$ motif to interact with other SFKs such as Lyn, Lck, Fyn and Src (65, 66, 68, 150), but its interaction with Hck shows the highest affinity (69). While HIV-1 Nef proteins mainly target Hck and Lyn, SIV Nef proteins prefer Fyn and Src due to the three amino acid changes within the "hydrophobic pocket" proximal to the SH3 binding domain (151).

Hck is specifically expressed in myeloid cells such as macrophages and neutrophils (152). The biological function of Hck is implicated in actin-dependent phagocytosis and migration (153). Nef promotes Hck catalytic activity by tightly binding to the SH3 binding domain of Hck (154). Dimerization of Nef seems to enhance Hck activation (155). It has also been shown that Nef activates endogenous Hck in myeloid TF1 cells regardless of Nef myristoylation status (156), thus it can be inferred that the majority of Hck activated by Nef may represent the cytoplasmic pool of Hck. Indeed, Hck is expressed in two isoforms p59 and p61 that localize differently within cells (157). Since the p59 isoform is more enriched in the plasma membrane than the p61 isoform, it is not surprising that the expression of active p59 and p61 triggers distinct functions; active p59 leads to membrane protrusion while active p61 changes the intracellular actin structure (158, 159). However, the significance of Hck activation for HIV-1 pathogenesis is currently unknown.

3. Macrophage and HIV-1 infection

The major target cells of HIV-1 infection are CD4+ T cells and macrophages. As the disease slowly progresses to AIDS, a dramatic depletion of CD4+ T cells causes opportunistic infection, tissue degeneration and ultimately host death. This highlights the leading role that CD4+ T cells play in HIV-1 infection. However, studies on target cells of macrophage lineage have demonstrated crucial roles for macrophages in HIV-1 infection.

The initial establishment of HIV-1 infection may rely on macrophages. During HIV-1 transmission, macrophages are among the first infected cells. Several reports have documented that in the seronegative patients with acute HIV-1 infection, virus isolation from blood is limited in monocytes but not in T cells (160, 161). Cultured blood monocytes were shown to support efficient propagation of some primary HIV isolates with high levels of progeny virus production (162). In line with these observations, most viruses seem to use CCR5 in the early stage of HIV-1 infection (107), probably suggesting a viral preference of targeting macrophages to initiate HIV-1 infection. Moreover, in certain tissues such as brain, lung and gut (163-165), macrophages represent the predominant HIV-1 infected cells.

Macrophages make a great contribution to the dissemination of HIV-1 virus particles. HIV-1 virus particles can be transmitted from macrophages to T cells in multiple ways. Viral dissemination can be mediated by cell-cell fusion between infected macrophages

and CD4+ lymphocytes or by the formation of a transit "virologic synapse" that rapidly transfers virus particles from infected macrophages to T lymphocytes (166). Alternatively, macrophages may also present virus particles to T cells in a similar way that APCs present antigens to activate helper T cells. In addition, macrophages specifically deliver virus particles to certain tissues. The HIV-1 infected circulating monocytes in blood are described as the "Trojan horse" that carry HIV-1 virus across the blood brain barrier (167). Once in the brain, they differentiate into macrophages and release virions. Similarly, infected circulating monocytes can travel to the gastrointestinal tract and differentiate into macrophages to initiate infection in intestine (168). Moreover, HIV-1 infection can be transmitted between individuals via bodily fluids such as semen and vaginal secretions in which the predominant cells are macrophages.

Macrophages maintain a major viral reservoir for continuous HIV-1 infection. Most HIV-1 infected T cells are killed by lytic HIV-1 infection. In contrast, HIV-1 infection in macrophages seems to occur in a milder way. Several mechanisms may account for the low level of HIV-1 cytopathicity in macrophages. For example, monocytes hardly have any cell surface CD4 molecules, and even after full differentiation, macrophages express much less cell surface CD4 than T cells. This low level of receptor expression not only limits initial viral entry but also prevents surperinfection. Indeed, the low level of post entry infection in macrophages is characterized by poor reverse transcription and a slower virus life cycle due to a limited dNTP pool (169). Low levels of HIV-1 infection in macrophages are usually associated with much less virus-induced cytopathic effect. Therefore, infected macrophages may still exist in the late stage of HIV-1 infection when

most CD4+ T cells are already depleted. In addition, HIV-1 infection triggers anti-apoptosis signaling in macrophages. The viral protein Nef, for example, is involved in several anti-apoptosis mechanisms: Nef-induced NF-κB activation is reported to inhibit TNFα-mediated apoptosis (170); Nef prevents apoptosis by inactivating pro-apoptosis factor Bad (171); Nef also affects the p53-mediated apoptosis pathway (172). Furthermore, macrophages not only survive longer in HIV-1 infection but also harbor virus particles. Virion assembly has been observed in the intracellular compartments of infected macrophages (173, 174), providing virus particles with a hidden storage place that is free of host immune responses. More importantly, it has been shown that even after macrophage infection is blocked for several weeks, the recovered intracellular virus particles can be transmitted to peripheral blood lymphocytes (175). Collectively, as long survivors in HIV-1 infection, macrophages maintain a hidden but continuous viral reservoir for HIV-1 infection.

Macrophages may also regulate HIV-1 infection by manipulating lymphocyte activities. Generally, macrophages provide innate immunity against foreign pathogens and amplify the immune response by activating cellular and humoral immunity. This is usually achieved through a variety of chemokines and cytokines that are produced by macrophages to regulate normal lymphocytes activities. In the case of HIV-1 infection, however, infected macrophages actually manipulate these regulatory signals for viral benefits, resulting in enhanced viral infection and damage to both T- and B-lymphocytes. Indeed, several HIV-1 proteins have the ability to induce cytokine and chemokine production during HIV-1 infection, which will be reviewed in detail in the chapter I-4.

Productive infection of T cells is hard to establish in tissues where only a limited amount of resting T cells are available. Nevertheless, a few HIV-1 infected macrophages are able to recruit as well as to activate target cells and therefore facilitate efficient virus spread in T cells (1). Infected macrophages also determine the fate of T cells. Models of selective killing of both CD4+ and CD8+ T cells by infected macrophages have been demonstrated. During HIV-1 infection, the expression of FasL and TNFα is upregulated on macrophages while their receptors are highly expressed on CD4+ and CD8+ T cells. Thus, death of T cells is regulated by infected macrophages through FasL- and TNFα-mediated apoptosis pathways, an indirect mechanism of T cell depletion that contributes to HIV-1 pathogenesis (176). In addition, infected macrophages are able to regulate HIV-1 latency in T cells. It has been shown that latent viruses in macrophages can be reactivated after CD40L stimulation, and interestingly autocrine secreted TNFα from activated macrophages also reactivates latent HIV-1 in bystander T cells (177), suggesting a possible mechanism of the reactivation of HIV-1 latency in T cells by macrophages. Moreover, infected macrophages mediate dysregulation of B cells. Recent findings have explained how the viral Nef protein expressed by macrophages causes dysregulated B cell activation. The hyperactivity of B cells has been linked to ferritin production induced by Nef in infected macrophages (178). However, another model shows that Nef can be transferred from macrophages to B cells through cell-cell contact and the presence of Nef in B cells leads to disruption of class-switch recombination and subsequent B cell dysfunction (179).

4. Cytokine and chemokine production during HIV-1 infection

Most viral infections trigger acute and strong immune responses in infected cells. These events constitute the first line of antiviral defense to reduce viral replication and to limit viral spread. One important antiviral event is the production of specific cytokines and chemokines by infected cells. The functions of these molecules are not limited to innate immunity but also stimulate the second wave defense of humoral and cellular immunity. Indeed, cytokines and chemokines regulate the distribution, activation, development and mortality of all kinds of immune cells.

Studies of HIV-1 infection have documented a long list of cytokines and chemokines, which includes but is not limited to IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-15, IL-16, TNF-α, TGF-β, IFN-α, IFN-β, IFN-γ, MIP-1α, MIP-1β, MCP-1 and RANTES (1, 180-199). (Note: MCP-1, MIP-1α, MIP-1β and RANTES are now known as CCL2, CCL3, CCL4 and CCL5, respectively. However, their original names are used in this thesis.) The effect of cytokines and chemokines on HIV-1 infection may be positive (IL-1, IL-6, TNF-α), negative (IL-10, IL-13, IL-15, IFN-α, IFN-β, RANTES), or dual (IL-4, IFN-γ, MIP-1α, MIP-1β) (1, 200, 201). Interestingly, a cytokine profile switch from Th1- to Th2- type, characterized by the loss of IL-2 and IFN-γ as well as the increase of IL-4 and IL-10 in the late stage of HIV-1 infection, seems to be correlated with AIDS development (202). In addition, a few cytokines have been reported as neurotoxic factors (203, 204). In summary, HIV-1 infection and disease progression is under profound regulation by complex signaling networks triggered by a variety of cytokines and

chemokines. The net effect, however, seems to result in more viral benefits than host defense. Indeed, several HIV-1 viral proteins such as Env, Tat, Vpr and Nef purposefully participate in the induction of specific cytokines or chemokines as well as in the manipulation of cellular signaling pathways, and thus convert the host antiviral machinery to viral advantage.

The gp120 unit of Env interacts with the receptor CD4 and the co-receptor CXCR4 and CCR5, which is already sufficient to trigger signaling pathways. It has been demonstrated that gp120 is able to activate all three mitogen-activated protein kinase (MAPK) family kinases ERK1/2, JNK and p38 in macrophages (205-207). Therefore, MAPK pathway activation might play a central role in gp120-induce signaling transduction. The upstream signaling events triggered by gp120-CD4 interaction and by gp120-CXCR4/CCR5 interaction may be different: gp120-CD4 interaction leads to the activation of two kinases Lck and Raf-1 through a Ras-dependent signaling pathway (208), whereas gp120 engagement with CCR5 activates kinases such as Lyn, PI3K and Pyk2 (209-211). The central downstream event of MAPK activation seems to be the release of TNF-α (212), one of the most potent NF-κB activators. Subsequent NF-κB activation may induce the production of other cytokines and chemokines. The full list of gp120-induced cytokines and chemokines includes IL-1, IL-4, IL-6, IL-8, IL-10, IL-13, TNF-α, IFN-α, IFN-β, IFN-γ [182-187]. At present, an in-depth understanding of how gp120-activated signaling pathways are linked to the synthesis of each cytokine/chemokine is still missing.

Tat is another active inducer of a few cytokines and chemokines such as IL-1, IL-2, IL-6,

IL-8, TNF-α and MCP-1 [188-194]. The increased expression of IL-2 by Tat is regulated primarily at the transcriptional level. The expression of Tat activates the IL-2 promoter, and both the NF-κB and NF-AT sites of the promoter are required (192, 213). Similarly, Tat mediates the synergistic increase of MCP-1 promoter activation by enhancing the binding activities of SP1, AP1 and NF-κB (189). In addition, Tat is also able to enhance the DNA binding activity of NF-κB, which increases IL-6 expression (214). These results suggest a central role of NF-κB in Tat-mediated induction of cytokines and chemokines. In fact, Tat is able to activate NF-κB via the IKK pathway, and PKC activation by Tat has been shown to facilitate the nuclear translocation of NF-κB (215, 216).

Vpr seems to have a dual effect on cytokine/chemokine regulation. An early finding showed that Vpr suppressed expression of IL-2, IL-4, IL-10, IL-12, TNF-α by inhibiting NF-κB activation via I-κB in T cells (217). The Vpr-induced suppression of IL-12 but not IL-10 was observed in macrophages (218). In addition, extracellular as well as virion-associated Vpr was reported to down-regulate β-chemokines, including MIP-1α, MIP-1β and RANTES, in PBLs and macrophages (219). In contrast to these suppressive effects, other results demonstrated that the activation of NF-κB and C/EBPβ by Vpr increased the expression of IL-8 in primary T cells and macrophages, as well as the expression of IL-6, IL-10, TNF-α and IFN-γ in a variety of cell types (193). In addition to the cytokine production regulated by Vpr, the production of β-chemokine RANTES in microglial cells was also induced by HIV-1 infection in a Vpr- and Nef- dependent manner (220). The regulatory function of Vpr on cytokine and chemokine production seems to be

determined by the dual effect of Vpr on NF-κB activation. Therefore, it is of great interest to study how Vpr manages to manipulate the bidirectional switch of the NF-κB signaling pathways.

Among all the HIV-1 viral proteins, Nef is probably the most competent regulator of cytokine and chemokine production. Nef is predominantly cytoplasmic and about 30% of Nef protein is membrane-associated. Although the concept of extracellular Nef is still controversial, Nef has been shown to be packaged into virions during assembly and may be secreted in exosomes (221). Nevertheless, despite the existing forms of Nef, suppression and induction of cytokines/chemokines by Nef have been widely reported in various cell types through distinct mechanisms. A simple regulation by Nef may not necessarily involve direct activation of signaling pathways. For example, Nef down-regulates MHC-I on APC and reduces CTL activation, which indirectly results in cytokine suppression in CTLs. Another example is that Nef can regulate membrane-associated cytokines such as TNF molecules via its ability to affect their internalization rate (222).

However, it is believed that complicated signaling pathways activated by Nef are involved during cytokine/chemokine regulation and sometimes may cause contradictory effects. For example, it was found that the expression of Nef interfered with IL-2 induction at the transcriptional level in T cells (223), and this suppressive effect was also observed by another group who confirmed that Nef expression in T cells down-regulated IL-2 as well as IFN-γ without affecting IL-4, IL-9, IL-13, IL-8 and TNF-α expression

(224). However, opposite findings suggested that Nef expression in T cells enhanced IL-2 production via a PI3K-dependent pathway (225, 226). The discrepancy of the cytokine profiles might be simply due to the differences of cell cultures, Nef isolates and T cell stimulation methods, or more profoundly it might reflect a complex signaling network that Nef manipulates in T cells. In monocytic cells, Nef may have a more consistent upregulating effect on the secretion of cytokines and chemokines. A few studies using recombinant Nef (rNef) have shown that extracellular Nef is sufficient to induce cytokines and chemokines (197). In a rat model, rNef, rather than Tat or gp120, induced production of IL-6, TNF-α and IFN-γ and was able to provoke leukocyte recruitment into the central nervous system (CNS) (227). The induction of IL-10 by rNef has been shown to rely on a calmodulin-dependent pathway (196). In addition, rNef selectively upregulated IL-15 synthesis in the macrophage population, which induced the activation and proliferation of unstimulated PMBCs, as well as the increased production of IFN-γ, TNF-α and IL-6 (194). However, it is not fully understood how extracellular Nef triggers signaling pathways and to what extent this represents the natural biological events mediated by Nef during HIV-1 infection. Presumably, extracellular Nef may bind to a cell surface receptor or may somehow enter the cells to initiate signaling. At present, the latter seems possible since it has been reported that rNef is efficiently internalized by macrophages and induces release of cytokines and chemokines such as IL-1, IL-6, TNF-α, MIP-1α and MIP-β, and the Nef-dependent cytokine/chemokine release is associated with NF-κB activation (197). IκB and all three MAPKs (ERK1/2, JNK and p38) are activated in this rNef-activated pathway (228). In agreement with this, rNef also induces

activation of NF-κB and AP-1 in U937 monocytic cell line through IκB degradation and JNK activation (229).

In comparison to results involving treatment with rNef, endogenous expression of Nef is a less artificial way to mimic the natural activities of Nef especially in the context of viral infection. The endogenous expression of Nef actively induces increased secretion of cytokines and chemokines in monocytic cells. However, the profiles of induced cytokines may vary depending on cell types. For example, expression of Nef in astrocytes mainly induced cytokines such as IL-2, IL-8, TNF-α as well as a chemokine MCP-1 (230), whereas the expression of Nef in DCs induced IL-6, IL-12, TNF-α as well as two chemokines MIP-1α and MIP-1β (198). Previous studies from our lab and others have focused on the manipulation of cellular signaling pathways by endogenous Nef in macrophages. The expression of Nef in HIV-1 infected macrophages induces a variety of secretory factors. Among them, two chemokines MIP-1α and MIP-1β appear to have a chemotactic effect on T cells (1) and probably on monocytes and macrophages as well. With more target cells attracted to sites of infection, HIV-1 may have a better chance to spread. HIV-1 replication in resting T cells is highly restricted, but endogenous Nef in macrophages has been shown to induce sCD23, sICAM and other unknown factors that eventually mediate the activation of resting T cells and make them permissive for viral replication (231). Moreover, the viral pathogenesis in B cells characterized by hyperactivation has been linked to ferritin expression in Nef-expressing macrophage supernatants (178). In conclusion, the dysregulated production of these regulatory

molecules indicates the ability of Nef to hijack the cellular signaling pathways of infected macrophages for viral benefits. However, the exact molecular mechanism involved in this Nef activity is unclear.

5. Study aims

The studies conducted in this thesis mainly attempt to identify the functional motif in Nef that is responsible for chemokine induction in macrophages as well as to clarify the relevance of this motif to other Nef functions. In addition, the role of Env in Nef-induced MIP-1β chemokine production in macrophages was determined. Distinct signaling pathways that regulate chemokine release by macrophages will be discussed. Finally, some preliminary results will be presented from the co-immunoprecipitation experiments performed to identify possible cellular factors that may be involved in chemokine regulation by Nef.

CHAPTER II.

MATERIALS AND METHODS

1. Cell cultures (III, IV, V)

Populations of monocytes were isolated by countercurrent centrifugal elutriation of mononuclear leukocyte-rich fractions of blood cells from normal donors undergoing leukopheresis (162). In 24-well plates, monocytes were as adherent cell monolayers cultured in DMEM (Gibco) medium containing monocyte colony stimulating factor (MCSF) (R&D system) for 2 days. Stimulated monocytes were then cultured in MCSF-free medium for another 5 days prior to use in experiments. Elutriated PBLs were activated with PHA (4 mg/ml) and were cultured in RPMI containing 10% human serum and 64 U/ml of interleukin-2 (ICN).

293T cells and TZM-bl cells were maintained in DMEM containing 10% Fetal Bovine Serum (FBS). Jurkat Tag Cell line was adopted from the lab of Dr.Heinrich Gottlinger and cells were maintained in RPMI containing 10% Fetal Bovine Serum (FBS).

2. Plasmid constructs (III, IV, V)

2.1 Proviral clones

HIV-1 NL4.3 clone was obtained from the AIDS Reagent Program of NIAID, NIH. In this thesis study, ADA clone was a modified NL4.3 variant harboring the NdeI (6399) - BamHI (8465) envelope fragment from the M-tropic primary isolate (Fig. 2-1). ADA-ΔNef was generated by replacing the first 237bp of the Nef gene in ADA with a unique

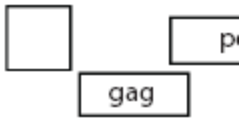

Figure 2-1: Construction of ADA, ADA-ΔNef and ADA-Nef.GFP fusions. The ADA clone was a modified NL4.3 variant harboring the NdeI (6399) - BamHI (8465) envelope fragment from the M-tropic primary isolate. ADA-ΔNef was generated by replacing the first 237bp of the Nef gene in ADA with a unique SrfI site, which was then used to subclone Nef-GFP fusion constructs.

SrfI site, which was then used to subclone GFP, wild-type Nef, Nef mutants or Nef-GFP fusion constructs (Fig. 2-1).

The reporter virus clone pNL4-3.Luc.R-E- was obtained from the AIDS Reagent Program of NIAID, NIH. This clone is Nef-, Env- and Vpr- because it has a luciferase gene inserted in the position of Nef as well as frameshifts at 5' of Env and the 26th amino acid of Vpr.

HIV-1 NL4.3 N- is a Nef deficient clone with a frameshift mutation at the XhoI site of the Nef gene. HIV-1 NL4.3 E- is an Env deficient clone with a frameshift mutation at the 5' of the Env gene. HIV-1 NL4.3 N-E- has both frameshift mutations in the Nef and Env genes.

2.2 Expression vectors

The SRα expression vector and the SRα-Nef plasmids were precious gifts from Dr.Heinrich Gottlinger, Umass Medical School. Point mutations K^{92}EK/3A, W^{57}L/AA, E^{62}EEE/4A were introduced into the SRα-Nef plasmid with the Quikchange II Site-Directed Mutagenesis Kit (Stratagene).

3. PCR-based mutagenesis (III, IV, V)

In this thesis, Nef gene mutants were generated by PCR-based mutagenesis methods. The 5'- and 3'- truncated Nef genes were directly amplified by PCR from pNL-4.3 using specific primers. A set of primers were designed so that the gene products had successive

15 amino acid deletions up to 90 amino acids from either N or C-terminus of Nef. For the convenience of western blotting analysis, all 3'- primers have extra coding sequences for an HA tag on the C-terminus.

Nef-GFP fusion constructs were made by fusing GFP to the C-terminus of Nef or Nef mutants and an HA tag was inserted as a linker for the convenience of western blotting analysis. The gene fusion constructs were amplified by overlapping PCR reactions and then subcloned into the SrfI site of the ADA-ΔNef.

Nef point mutations $K^{92}EK/3A$, $R^{105}R/AA$, K^{92}/A, E^{93}/A, K^{94}/A, $D^{174}D/AA$, $E^{155}E/AA$ were introduced into a pcDNA-Nef plasmid with the Quikchange II Site-Directed Mutagenesis Kit (Stratagene). Mutated genes were confirmed by sequencing, amplified by PCR and inserted into the SrfI cloning site of the ADA-ΔNef.

4. Virus preparation and macrophage infection (III, IV, V)

To make virus stocks, 293T cells were transfected with proviral clones using the TransIT transfection kit (Mirus). Pseudotyped viruses were produced by co-transfecting vectors expressing VSV-G, YU2 Env and NL4.3 Env. Fresh medium was changed 6 hours post transfection. 48 hours later, virus-containing supernatants were harvested and filtered with 0.22μ conical tubes (Millipore). Virus titers were normalized by reverse transcriptase (RT) activity.

Monocyte-derived-macrophage (MDM) cells were seeded in 24-well plates (7×10^5/well) and were incubated with virus stocks (2-5×10^6 cpm/well) overnight. The incubation time for VSV-G-pseudotyped virus inoculation was 2-4 hours. Viruses were then removed and replaced with fresh medium after cells were extensively washed. Infected cells were incubated at 37°C and at indicated time points 500μL supernatants from infected cultures were collected and 500μL fresh medium was added back.

5. RT assay (III, IV, V)

To determine the titers of virus stocks, virus-containing supernatants were harvested at 48 hours post transfection, clarified by low-speed centrifugation, and filtered through 0.45μm-pore filters prior to RT assay. A 5μL sample was used for the RT assay. To monitor replication in infected macrophage, supernatants were collected at indicated time points and a10μL sample was used. RT activity was measured by quantification of ^{3}H-TTP labeled reverse transcribed products. Briefly, virions were disrupted by adding an equal volume of a solution A containing 0.1% NP-40, 10mM DTT, 300 mM KCl, 100 mM Tris-HCl (pH 7.9). Lysed virions were used for an overnight reverse transcription reaction in solution B with 10μCi/mL ^{3}H-TTP. Quantification of reverse transcribed products was carried out with the Microbeta 1450 scintillation counter (Perkinelmer).

6. ELISA (III, IV, V)

Quantikine ELISA kits (R&D system) for human MIP-1β, MIP-1α, IL-6, IL-16, MCSF, TNF-α and IP-10 were used according to the manufacturer's protocols to measure their

concentrations in 100μL macrophage supernatants. ELISA system for human ferritin was developed with ELISA plates coated with ferritin antibodies (DAKO) and HRP-conjugated secondary antibody (Fitzgerald Industries International, Inc.).

7. Western blotting analysis (III)

To facilitate western blotting analysis, an HA tag was fused to C-terminus of Nef mutants alone or in the context of Nef.GFP fusion products. Transfected cells were lysed in RIPA buffer with cocktail tablet of protease inhibitors (Roche). Cell lysates were analyzed by SDS-PAGE and western blotting with a mouse monoclonal anti-HA antibody HA.11 (Covance).

8. Assay of CD4 and MHC-1 down-regulation (IV)

Jurkat Tag cells were nucleofected with the SRα vector expressing wild-type Nef or Nef mutants as well as a GFP expressing vector at a molar ratio of 3:1 (Amaxa nucleofector, Lonza). Twenty-four hours post-transfection, cells were washed and stained in dark with PE-conjugated mouse anti-human CD4 or anti-human HLA-ABC antibodies (BD Pharmingen) for 30 minutes. Stained cells were washed three times and analyzed by FACS.

9. Neurotoxicity assay (IV)

We used the NT2.N cell line to generate a pure and reproducible neuron cell population (232). NT2.N neurons were plated on poly-D-lysine and Matrigel coated 96-well plates

(25,000 cells per well). After 7 days, neurons were exposed to conditioned medium from HIV-infected macrophages. 48 hours after addition of conditioned medium, neuronal apoptosis was measured by Caspase-Glo 3/7 Assay (Promega) according to the manufacturer's protocol.

10. Virus infectivity assay (IV)

We produced reporter viruses by co-transfecting 293T cells with the NL4-3.Luc.R-E- plasmid, a T-tropic or M-tropic envelope expression vector as well as the SRα plasmid expressing Nef or K92EK/3A mutant. Infectivities of M-tropic viruses were examined in TZM-bl cells and macrophages, while infectivities of T-tropic viruses were examined in Ghost cells and PBLs. Virus infectivities were determined by measuring luciferase activities in infected cells using Bright-Glo Luciferase Assay System (Promega).

11. Co-immunoprecipitation (IV, V)

293T cells were transfected with vectors expressing HA- and FLAG-tagged proteins using the TransIT transfection kit (Mirus). After 48 hours, transfected cells were lysed in the lysis buffer containing 0.5% n-octyl glucoside, 20mM tris HCl pH 7.5, 100mM $(NH_4)_2SO4$ and 20% glycerol. The lysates were centrifuged at 15,000rpm to spin down cell debris for clarification. The supernatants were immunoprecipitated with anti-HA antibody HA.11 (Covance). Immunoprecipitates were analyzed by SDS page and immunoblotting with antisera against the PRD of Dyn2 (Affinity BioReagents) or anti-FLAG antibody M2 (Sigma), or silver staining.

12. Reagents (V)

The CD40L was supplied by Alexis Corporation.

The anti- human CD4 antibody (clone RPA-T4) that was able to inhibit HIV-1 by blocking gp120 was purchased from BD Pharmingen.

The CCR5 antagonist TAK779 was a gift from Dr. Paul Clapham, Umass Medical school.

CHAPTER III.

A NOVEL MOTIF IN HIV-1 NEF THAT REGULATES MIP-1β CHEMOKINE RELEASE IN MACROPHAGES.

Abstract

All primate lentiviruses encode Nef, an accessory protein that is critical for viral pathogenicity *in vivo*. Multiple cellular functions of Nef have been described *in vitro* and important functional motifs for these functions have been identified. We have previously demonstrated that HIV-1 Nef regulates the release of chemokine MIP-1β from infected macrophages and have proposed that this may enhance conditions for viral replication by promoting recruitment of substrate lymphocytes to sites of infection. However, the Nef motif responsible for this function is unknown. In the current study, we first examined several previously identified Nef motifs but excluded their roles in chemokine induction by Nef. By mutagenesis analysis, we mapped the functional domain to a region between residue 84 and residue 116 of HIV-1 NL4.3 Nef. In this region, we identified a novel motif ($K^{92}EK$) that was required for Nef-dependent chemokine production by HIV-1 infected macrophages. Importantly, the KEK motif is functionally conserved in SIVmac239 Nef. This further underscores Nef-induced chemokine regulation as an important cellular activity in viral replication and pathogenicity.

Introduction

The Nef protein of primate lentiviruses is important for viral replication and pathogenicity *in vivo*. Patients infected with Nef-defective viruses have undetectable viral loads and do not exhibit clinical manifestations of AIDS (47, 233). Extensive studies have described distinct cellular functions that may explain the critical role of Nef in HIV-1 infection *in vivo*. The first characterized property of Nef is its ability to induce down-modulation of cell surface molecules such as CD4 and MHC-I, which results in enhanced viral replication as well as impaired host immune responses. Nef also enhances virus production and infectivity. It has been shown that Nef, in viruses lacking Vpu, appears to counteract a cellular restriction factor tetherin/BST2 and enhance virus budding (234, 235). Several groups have suggested that more infectious progeny virions are produced in the presence of Nef. Finally, Nef harbors multiple motifs that can potentially manipulate cellular signaling pathways through engagement of cellular proteins such as Hck, Pak2 and Vav (138, 236, 237). For most Nef functions described above, distinct functional motifs in Nef have been identified, which greatly contributes to in-depth understanding of the molecular mechanisms for the corresponding Nef functions.

A potential role of Nef in altering macrophages functions was first indicated in an early study with an SIV/macaque model in which a Nef variant confered on SIVmac239 the ability to replicate in resting PBMCs consisting of resting lymphocytes and monocyte/macrophages (238). Interestingly, although viral replication was found predominantly in resting T cells, it was highly dependent on the presence of macrophages.

How resting T cells in culture with macrophages became supportive of productive viral replication was not fully understood. The question has been addressed by our previous studies suggesting that macrophage is an important site of Nef action. Upon HIV-1 infection, macrophages release a variety of regulatory molecules; these include two CC-chemokines MIP-1α and MIP-1β that promote recruitment of T cells (1), sCD23, sICAM and probably other unknown factors that mediate activation of resting T cells (231), as well as ferritin that causes hyperactivation of B cells (178). Importantly, the viral protein Nef is necessary and sufficient to induce production of these molecules by infected macrophages. Since Nef has the ability to interact with cellular kinases, we have proposed a model in which Nef mediates secretion of regulatory molecules to enhance conditions for viral replication and dissemination by manipulating cellular signaling pathways in macrophages. This raises an essential question as what is the exact molecular mechanism of this Nef activity, and undoubtedly identification of the required motif in Nef is the first step to an answer.

Nef actually induces a wide range of regulatory molecules in macrophages. Some of them such as TNF-α were detected in one study but not in another (1, 197). However, potent induction of chemokine MIP-1β by Nef has been consistently reported indicating that it represents a reproducible activity of Nef that is least affected by different experimental conditions (1, 197, 199). Therefore, in this thesis, we mainly focused on MIP-1β induction in the context of viral replication as the major index of Nef-induced cytokine/chemokine production.

In this chapter, we first tested whether several previously identified Nef motifs were required for chemokine induction and the results showed that they were dispensable. The mutagensis analyses mapped the functional domain to a region between residue 84 and residue 116 of HIV-1 NL4.3 Nef. We next tested several point mutants in this region and identified a novel motif ($K^{92}EK$) that was required for chemokine induction. Moreover, the KEK motif was also functionally conserved in SIVmac239 Nef since corresponding mutations also impaired MIP-1β induction by SIVmac239 Nef. This further indicates Nef-induced chemokine regulation may be an important cellular activity in viral replication and pathogenicity.

Results

Nef induces MIP-1β production independently of other classical Nef activities.

Before the activity of Nef-regulated cytokine/chemokine production was recognized, the classical functions of HIV-1 Nef were characterized into three major categories: 1) down-regulation of cell surface molecules such as CD4 and MHC-I; 2) enhancement of virus infectivity; 3) interaction with cellular kinases such as Pak2 and Hck. Distinct motifs required for these functions have been identified (reviewed in chapter I). Some seem to be solely responsible for a single function. For example, $L^{164}L$ and $E^{62}EEE$ are required for down-regulation of CD4 and MHC-I, respectively, but are largely dispensable for enhancement of infectivity or engagement with Hck. However, mutations in myristoylation site G^2, dimerization site $P^{122}D$ and SH3 binding interface $P^{72}XXP$ impair many Nef activities.

The relevance of these classical Nef activities to chemokine induction is unknown. To address this, we examined MIP-1β chemokine induction by several representative Nef mutants, G^2/A, $P^{122}D/AA$, $L^{164}L/AA$, $E^{62}EEE/4A$ and $P^{72}XXP/2A$, which were defective in at least one or more classical Nef functions. Macrophages were infected with macrophage-tropic recombinant viruses that harbor Nef mutations as indicated along with wild-type and ΔNef viruses (Fig. 3-1). Although all viruses exhibited robust growth in macrophage culture, the wild-type Nef virus induced at least 10-fold higher MIP-1β

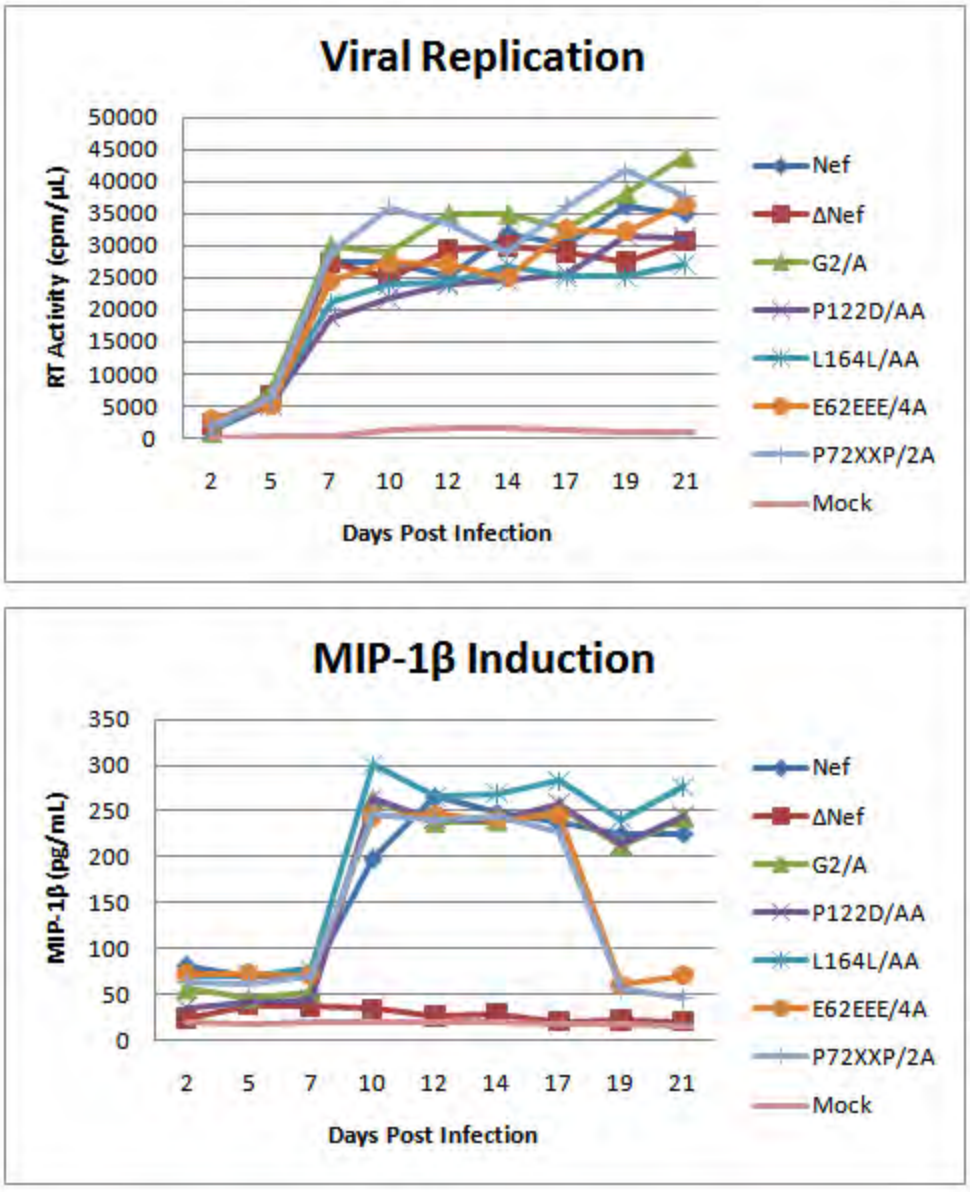

Figure 3-1: Nef induces MIP-1β production independently of its other classical functions. Macrophages were infected by HIV-1 recombinant viruses harboring wild-type Nef and Nef with indicated point mutations. Upper panel, viral replication was gauged from levels of reverse transcriptase (RT) activity in culture supernatants. Lower panel, MIP-1β levels were determined by ELISA (Jacque, Unpublished data).

production than the ΔNef virus or mock infection. Similarly, all Nef mutant viruses were able to induce 9-fold to12-fold higher MIP-1β production than ΔNef virus. The corresponding mutated motifs appeared to be dispensable for chemokine induction by Nef. Therefore, we concluded that MIP-1β chemokine production can be induced by Nef independently of other classical Nef activities.

The domain required for MIP-1β induction maps to a 31-amino acid region in HIV-1 NL4.3 Nef

With the motifs responsible for other classical Nef functions excluded, it was speculated that Nef regulated MIP-1β chemkine production through some unknown motifs. To identify the possible motifs, we narrowed down the Nef region that was required for MIP-1β induction by deletion mutagenesis.

To determine whether the functional motifs lay in the N or C-terminal half of the protein, a series of Nef truncations with successive 15 amino acid deletions up to 90 amino acids from either the N or C-terminus were constructed (Fig. 3-2). An HA tag was inserted at the C-terminus of the truncated proteins in order to facilitate their analysis by western blotting. The result shows that truncations of up to 30 amino acids from the N-terminus or up to 45 amino acids from the C-terminus were stably expressed but longer truncations were unstable. To facilitate the analysis of unstable Nef mutants with longer truncations, GFP protein was fused to the C- terminus of Nef truncation mutants lacking 90 amino acids from either the N or the C-terminus (NΔ90.GFP and CΔ90.GFP, respectively).

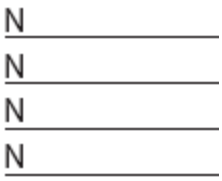

Figure 3-2: Construction of Nef mutants containing stepwise 15-amino acid truncations from the N or C-terminus of HIV-1 Nef. Expression of a series of Nef truncation mutants was evaluated by western blotting with an antibody directed to an HA-tag at the C-terminus of the Nef mutants.

These Nef-GFP fusions were next inserted into a macrophage-tropic ADA-ΔNef clone in the place of Nef (Fig. 2-1). The Nef-GFP fusion proteins lacking 90 amino acids from the N- or C-terminus were stably expressed (Fig. 3-3, lower panel). Monocyte-derived macrophages were infected with these recombinant viruses and culture supernatants were monitored at two- or three-day intervals for the presence of viral replication by RT and the level of MIP-1β by ELISA (Fig. 3-4). All recombinant viruses underwent a robust spreading infection in macrophage cultures and MIP-1β induction was evident in macrophages harboring a wild type Nef (Nef) or Nef fused to GFP at the C-terminus (Nef.GFP) (Fig. 3-4, left panels). There was no chemokine induction in macrophages infected with the virus lacking Nef (ΔNef) or in a virus harboring GFP in place of Nef (GFP). Interestingly, chemokine induction was also evident in macrophages infected with HIV-1 variants harboring a 90 amino acid truncation at either the N or the C-terminus (NΔ90.GFP, CΔ90.GFP, respectively) (Fig. 3-4, right panels). Therefore, the first and last 90 amino acids of Nef appeared to be dispensable for the induction of chemokines in infected macrophages.

Residues 91-116 in the conserved core region of Nef were the only sequences not affected by 90 amino acid truncations from the N- and C-terminus. To examine the role of this region in chemokine induction, 115 amino acids from the N- and C-terminus (NΔ115, CΔ115, respectively) were truncated and fused to GFP (Fig.3-4, upper panel). Additionally, an internal deletion (NefΔ85-115) removing 31 amino acids from the Nef core domain was constructed (Fig.3-3, upper panel). Stable expression of these Nef-GFP

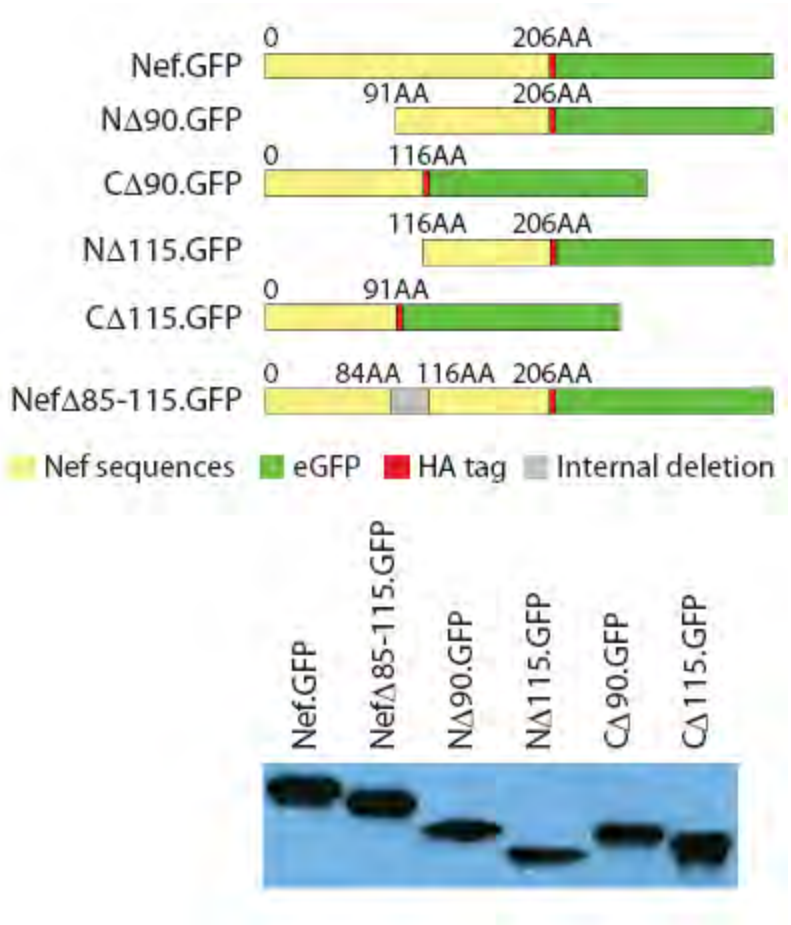

Figure 3-3: Construction and expression analysis of Nef-GFP fusion proteins. Upper panel, outline of Nef-GFP fusion constructs harboring 90 or 115 amino acid truncations from the N or C-terminus or harboring an internal 31 amino acid deletion. Lower panel, expression of Nef-GFP truncation and deletion mutants was analyzed by western blotting with an anti-HA antibody.

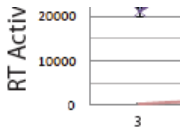

Figure 3-4: Analysis of chemokine production in macrophages infected by HIV-1 variants harboring wild-type Nef and truncation/deletion mutants with GFP fusion. Left panels, viral replication was gauged from levels of reverse transcriptase activity in culture supernatants. Right panels, MIP-1β levels were determined by ELISA. Results are shown for macrophages cultures obtained from three independent donors.

fusions was confirmed by western blotting analysis (Fig.3-3, lower panel). In contrast to viruses harboring 90 amino acid truncations at the N- or C-terminus of Nef, viruses harboring 115 amino acid deletions at the N- or C-terminus, or the virus containing a 31 amino acid deletion between residues 84 and 116 of Nef, were unable to induce MIP-1β (Fig. 3-4). This pattern was consistent in macrophage cultures prepared from three independent donors. These results demonstrated that a region located between residue 84 and residue 116 of HIV-1 NL4.3 Nef was necessary for MIP-1β chemokine induction.

The KEK motif is responsible for MIP-1β chemokine induction in HIV-1 infected macrophages.

Both HIV-1 Nef and SIV Nef induce chemokine production in infected macrophages (239), suggesting the presence of a functional motif that is common to HIV-1 and SIV Nef proteins. Alignment of the HIV-1 NL4.3 Nef region required for chemokine induction with the analogous sequences of SIVmac239 Nef revealed some candidate motifs (Figure 3-5). We focused our analysis on two motifs, $K^{92}EK$ and $R^{105}R$, since these are predicted to be surface residues with the potential to interact with cellular factors (63, 66). Nef mutants with alanine substitutions at $K^{92}EK$ ($K^{92}EK/3A$) and $R^{105}R$ ($R^{105}R/AA$) motifs were inserted into HIV-1 ADA-ΔNef and examined for the ability to induce MIP-1β in infected macrophage (Fig. 3-6). Chemokine induction by the virus expressing wild-type Nef and the virus lacking functional $R^{105}R$ motif ($R^{105}R/AA$) were indistinguishable (Fig. 3-6). In contrast, MIP-1β levels in macrophage cultures infected with the Nef mutant lacking the $K^{92}EK$ motif ($K^{92}EK/3A$) were indistinguishable from

Figure 3-5: Alignment of HIV-1 NL4.3 and SIVmac239 Nef sequences. Common residues are shown in blue and motif candidates with predicted surface accessibility in green. Corresponding consensus sequences in different HIV-1 and SIV clades are listed in the box.

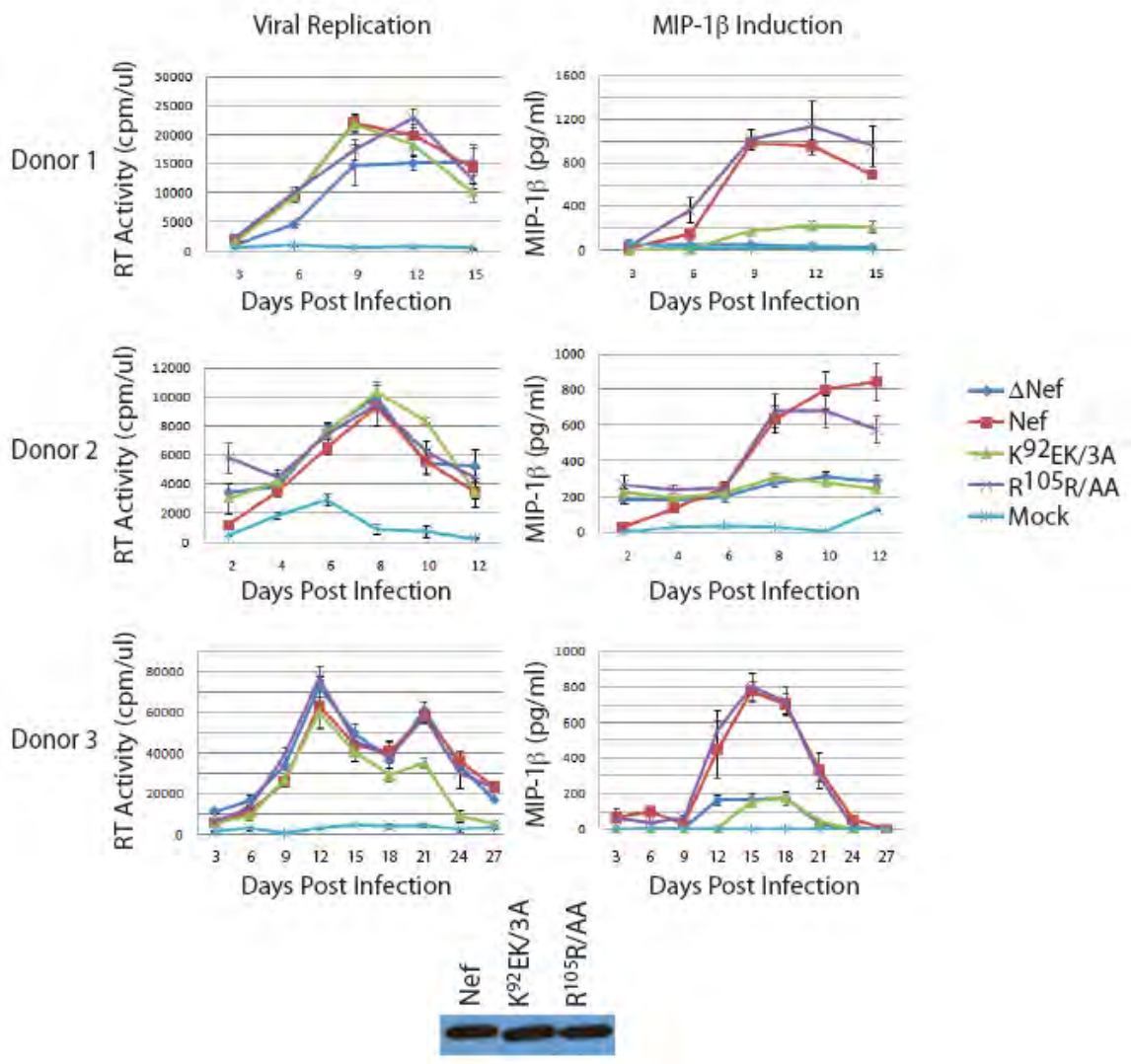

Figure 3-6: Identification of the $K^{92}EK$ motif within HIV-1 Nef as the determinant for MIP-1β induction in infected macrophages. Upper panel, viral replication and MIP-1β production in macrophages infected with viruses harboring wild-type Nef and Nef variants containing amino acid substitutions in the core domain of Nef. Results are shown for macrophages from three independent donors. Lower panel, expression levels of Nef alleles harboring the indicated alanine substitutions was confirmed by western blotting.

macrophages infected with a ΔNef virus (Fig. 3-6). Consistently, this pattern was observed in macrophage cultures derived from three independent donors. Therefore, we conclude the $K^{92}EK$ of HIV-1 NL4.3 Nef represents a functional motif that is responsible for MIP-1β induction. Furthermore, the KEK motif seems to be well conserved in most clades of HIV-1 and SIV (Fig. 3-5), and more importantly, when the KEK motif in SIVmac239 Nef was substituted with alanines, MIP-1β production was also impaired relative to virus harboring a wild-type SIV Nef in HIV-1 infected macrophages (Fig. 3-7). These further underscored a critical role of the KEK motif in chemokine induction by distinct Nef alleles.

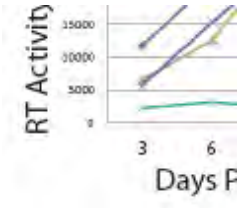

Figure 3-7: Role of the KEK motif in chemokine induction by SIV Nef. Macrophages were infected with HIV-1 viruses expressing wild-type SIVmac239 Nef or SIVmac239 KEK mutant. Viral replication and MIP-1β production were measured by RT assay (left panel) and ELISA (right panel), respectively.

Discussion

In this chapter, we reported the identification of a novel functional motif KEK in HIV-1 and SIV Nef that was responsible for induction of chemokine MIP-1β in infected macrophages. The mutagenesis analysis, which led us to the location of this motif, was carried out in three stages.

In the first stage, we tested five previously reported Nef point mutations including G^2/A, P^{122}D/AA, L^{164}L/AA, E^{62}EEE/4A and P^{72}XXP/2A for their ability to regulate chemokine induction in HIV-1 infected macrophages. The results showed that all the mutants were as functional as the wild-type Nef in terms of MIP-1β induction, thus indicating these mutated motifs were not responsible for chemokine induction. These motifs have been shown to be required for classical Nef functions including down-regulation of CD4 and MHC-1, enhancement of virus infectivity and interaction with cellular kinases, which represent the most fundamental cellular activities currently known about the Nef proteins. However, exclusion of these motifs may have some implications in the exact mechanism of Nef-induced chemokine induction.

The N-termianl G^2 of HIV-1 Nef is the myristoylation site, which is strictly required for membrane targeting of Nef. The G^2/A mutation which prevents myristoylation has been shown to impair most Nef functions. Interestingly, the G^2/A mutant was still highly active in MIP-1β induction and the production induced by G^2/A was indistinguishable

from that by wild-type Nef. Thus, myristoylation and membrane attachment may be dispensable for chemokine induction by Nef. This is supported by the results from other studies showing that unmyristoylated Nef induces chemokines in macrophages (197). Although about 30% of myristoylated Nef is attached to the plasma membrane, a large pool of Nef is present abundantly in the cytoplasm of infected cells. However, contribution of this cytoplamic Nef population to the cellular functions of Nef has been largely ignored. Our results from the G^2/A mutant provide new evidence for possible cellular activities of cytoplasmic Nef, perhaps by manipulating cellular signaling pathways.

The motif critical for HIV-1 Nef to form dimers was mapped to P^{122}D (90). Mutation of this motif abolished CD4 and MHC-1 downregulation as well as the infectivity enhancement by Nef, suggesting that dimerization of Nef was important for these Nef activities. In contrast, chemokine induction by Nef was independent of the dimer form of Nef, since the P^{122}D/AA mutant induced a high level of MIP-1β production. Together with the information from the G^2/A mutant, this may suggest a flexible structural requirement for Nef to induce chemokines in infected macrophages.

Nef down-regulates CD4 and MHC-I by different mechanisms. In the case of CD4, Nef mediates the internalization and degradation of cell surface CD4 molecules by directing them to the clathrin adaptor AP-2 (63, 85, 86). Nef induces MHC-I down-regulation through the PACS-dependent sorting pathway. The Nef mutant L^{164}L/AA loses its association with AP-2, while the E^{62}EEE/4A fails to bind to PACS-2 (85, 95), and thus

are defective in CD4 and MHC-I down-modulation, respectively. The ability of these two mutants to induce MIP-1β production indicates that the Nef-mediated lysosome or endosome pathways are uncoupled from Nef-induced chemokine induction.

In the case of MHC-I down-regulation, Nef binds PACS-2 through the E^{62}EEE motif and the complex triggers a multi-kinase cascade that eventually accelerates MHC-I endocytosis. In this signaling pathway, Nef needs another motif P^{72}XXP to bind and activate SFK in TGN and initiates signal transduction (95). In fact, this proline-rich motif also mediates the interaction between Nef and intracelluar kinases such as Pak2 and Hck. In a multi-protein signalosome that also contains Rac, Cdc42 and Vav1 (240), Nef activates Pak2, which mediates downstream effects. Phosphorylation of at least one substrate kinase merlin, a key signaling molecule, by the Nef-Pak2 complex has been confirmed in primary T cells (241). Therefore, the asscociation of Nef with Pak2 appears to be central to alter signaling pathways in T cell activation (242). Similarly through the P^{72}XXP motif, Nef tightly binds Hck, a Src family kinase primarily expressed in cells of macrophage lineage. The biological significance of the interaction between Nef and Hck in macrophages is unclear. It has been shown to negatively regulate cell surface expression of the MCSF receptor Fms on macrophage (243) as well as to promote fibroblast transformation (244). However, there is relatively little evidence for an essential role of the Nef-Hck interaction in priming macrophage activation. In monocytic cell lines, constitutive production of active transcription factor AP-1 by Nef appears to be dependent on the MAPK pathway and can be blocked by expressing dominant-negative Hck, suggesting that the Nef-Hck interaction may trigger signaling events via the MAPK

pathway that leads to AP-1 induction in macrophages (245). Therefore, it was tempting to speculate that the Nef-Hck interaction might be responsible for the Nef-dependent chemokine induction in macrophages. Our results, however, clearly demonstrate that the P^{72}XXP/AA mutant defective in Hck interaction still remains fully active for MIP-1β induction, which excludes the possibility that chemokine induction by Nef in macrophages is solely dependent on its interaction with Hck. More importantly, it indicates unknown signaling molecules other than Hck that Nef might target for signal transduction in infected macrophages.

In the second stage, we utilized a progressive truncation strategy to identify the region that harbored the determinant for chemokine induction in Nef. We initially constructed a series of 15-amino acid deletions at both the N- and C-terminus of HIV-1 NL4.3 Nef. Analysis of these mutants was hampered by the fact that deletions of more than 30 amino acids from the N-terminus or more than 45 amino acids from the C-terminus resulted in truncated mutants with undetectable expression level due to poor stability. This finding is consistent with the previous work of others indicating that the N- and C-terminal regions of Nef are critical to stabilize Nef structure (132, 142, 246). Importantly, our result further defined the truncation limits in both terminal regions of Nef that can be tolerated for stable expression. The regions, aa30-45 in the N-terminus and aa 45-60 in the C-terminus, appeared to be critical, since mutants with truncations in these regions were unstable. The importance of these regions in structural stablization is supported by Nef structures shown in NMR and crystallography. Most of the N-terminus of Nef does not have a folded structure. Nevertheless, there is a well-defined short α-helix between

Arg35 and Gly44 that is further stabilized by myristoylation (64). Our results of the unstable N-terminal truncation mutants, along with others (38, 84), underscore an important role for this α-helix in Nef stability. In additon, our results from the unstable C-terminal truncation mutants are perfectly consistent with the previous work in which HIV-1 SF2 Nef was shown to lose stability when more than 40 amino acids were deleted from the C-terminus (142). Unlike the N-teriminal aa30-45 region with α-helical structure, the aa45-60 region in the C-terminus was actually located in a flexible loop. Therefore, mutation in this region should not impair the general structural organization of Nef. An alternative explanation might be the internal PEST-like sequences in Nef that, in the C-terminal truncated mutants, moves closer to the C-terminus and thus may direct the truncated products for premature degradation (247).

The GFP fusion facilitates further analysis of unstable truncated Nef mutants that have large deletions in the N- and C-termini. Other groups have utilized the GFP fusion strategy to study the localization of Nef under distinct circumstances and it has been confirmed that fusing Nef at the C-terminus with GFP helps to avoid artificial localization of the Nef fusion proteins (80, 242, 248). As expected, ΔN90.GFP and ΔN115.GFP were present exclusively in the cytoplasm due to the abscence of plasma membrane targeting sequence, whereas ΔC90.GFP and ΔC115.GFP were localized to the cell membrane but also abundant in the cytoplasm (Dai, unpublished data). It should be noted that ΔN90.GFP and ΔC90.GFP both actively induced MIP-1β in macrophages despite the difference in localization. This is consistent with the result obtained with the

G2/A Nef mutant and further confirms the role of the cytoplamic pool of Nef proteins in chemokine induction.

Furthermore, GFP fusion stabilized the truncated Nef mutants and thus significantly enhanced the expression of Nef.GFP fusion proteins as confirmed by western blotting analysis. However, relatively lower expression of ΔN90.GFP and ΔN115.GFP than others indicated that the N-terminus of Nef was a more critical structural determinant than the C-terminus (Fig. 3-3). Interestingly, ΔN90.GFP, although expressed at a lower level than ΔC115.GFP, was able to induce a significantly higher level of MIP-1β production. Therefore, it was the Nef determinant within the deletion region, rather than the expression level, that regulated the chemokine induction in macrophages. In addition, it has been demonstrated that the Nef.GFP fusion protein is defective in Pak-2 activtition (249), and since we have shown in our study that Nef.GFP was fully functional in MIP-1β induction, this gives another line of evidence, in addition to the ability of the P^{72}XXP/2A and R^{105}R/AA mutants to induce MIP-1β, which indicates that Pak-2 activation is not involved in chemokine induction by Nef.

In the last stage, we introduced point mutations into two candidate motifs including K^{92}EK and R^{105}R, within the Nef region that was indispensable for chemokine induction and identified K^{92}EK as the functional motif. The selection of these candidates was based on two conditions. Firstly, they are all conserved residues in HIV-1 and SIV Nef. Since SIV Nef has been shown to induce MIP-1β as well (198), we were most interested in motifs consisting of residues common to HIV-1 and SIV Nef. In addition, most of these

motifs, according to Nef structural analysis, are proposed to have surface accessibility for potential interaction with cellular proteins (63, 66). This is important because Nef itself has no known enzymatic activity, and therefore signaling pathways triggered by Nef most likely involve its interaction with cellular proteins. Indeed, the $R^{105}R$ motif has been reported to be responsible for such Nef activities. Nef must have an intact $R^{105}R$ motif to bind and activate Pak-2 (143). Our result showed the $R^{105}R$/2A mutant was fully functional for MIP-1β induction, which also supports the idea that Nef induces chemokines in HIV-1 infected macrophages independently of other biological activities. Among all the point mutants we tested, $K^{92}EK$/3A consistently failed to induce MIP-1β production in macrophages cultures prepared from three independent donors. Notably, alanine substitution of the corresponding KEK residues in SIVmac239 Nef also impaired its ability to induce MIP-1β production and the KEK motif is highly conserved in distinct Nef isolates from HIV-1 and SIV clades, suggesting KEK motif-dependent chemokine induction may represent a conserved critical function of SIV and HIV-1Nef in macrophages.

In our experiments, wild-type Nef or Nef mutant proteins were expressed endogeneously in the context of viral infection. We collected supernatants at various time points to monitor viral replication and chemokine production. Interestingly, by comparing the curves of viral replication and corresponding chemokine production, it appeared that the peak of the MIP-1β chemokine induction usually correlated with that of the viral replication. In some donors, the chemokine curves reached the peaks even two or three days later than the viral replication curves. In addition, in some experiments when

macrophages failed to support spreading infection, MIP-1β was not detected even for the wild-type virus (Dai, unpublished data). These results indicate that Nef-induced chemokine production is viral replication dependent, which is supported by another study showing that inactivated, noninfectious virus particles do not induce chemokine secretion from DCs (198).

Chapter IV.

Impact of the $K^{92}EK/3A$ mutation on distinct Nef functions

Abstract

By mutagenesis analyses, we identified the KEK motif in Nef as the determinant that regulated MIP-1β chemokine production by HIV-1 infected macrophages. This novel motif had not been linked to any other Nef activities. Thus, it remained to be elucidated whether mutations in the KEK motif affected other Nef functions. In this chapter, we attempted to examine the cellular effects of the HIV-1 Nef mutant K^{92}EK/3A in terms of 1) down-regualtion of CD4 and MHC-I; 2) induction of secretory factors; 3) neurotoxicity; and 4) enhancement of virus infectivity. The K^{92}EK/3A mutant exhibits normal down-regulation of both CD4 and MHC-I molecules. In the supernatant from macrophages infected by K^{92}EK/3A mutant virus, the production of several other regulatory factors such as MIP-1α, IL-6, IL-16 and ferritin was also impaired. The conditioned macrophage supernatants from K^{92}EK/3A mutant virus infection exhibited less neuronal toxicity than that from wild-type virus infection. Finally, the K^{92}EK/3A mutant had no significant effect on virus infectivity in macrophages and PBLs. These results suggest a specific role of the KEK motif in Nef functions that may contribute to viral replication and pathogenicity.

Introduction

In infected macrophages, Nef induced chemokine production in a way that appeared to be independent of other cellular activities of Nef, as suggested by the normal MIP-1β level induced by Nef point mutants defective in other Nef functions (Fig.3-1). The mutagenesis analyses identified the Nef mutant motif K^{92}EK/3A that is defective for MIP-1β induction in infected macrophags. We have demonstrated that the K^{92}EK/3A mutant appeared to be as stable as the wild-type Nef by western blotting analysis (Fig. 3-6). However, it is still possible that mutations in KEK motif leads to a global impact on Nef that may impair all cellular activities of Nef, which may not be detected simply by western blotting analysis. In this chapter, we aimed to determine how mutations in the KEK motif influence other Nef activities.

Although the down-modulation of CD4 and MHC-I molecules are two independent activites of Nef, they generally represent the hallmark cellular activities of Nef. Their mechanisms are relatively well established and a few defective Nef mutants in these functions have been described. Mutations in L^{164}L and E^{62}EEE lead to specific functional loss of CD4 and MHC-I downregulation, respectively. In contrast, mutations in G^{2} and P^{122}D impair these Nef functions in a non-specific way that also affects other Nef functions as well. Although Nef mutants L^{64}L/2A and E^{62}EEE/4A exhibit full activity for MIP-1β induction indicating that CD4 and MHC-I downregulation were not required for chemokine induction, it was also important to address whether the K^{92}EK/3A mutant defective in chemokine induction was still functional in CD4 and MHC-I down-

regulation.

We have shown that the KEK motif was indispensable for MIP-1β induction in macrophages. In those experiments, we measured MIP-1β as the major standard for the ability of Nef or Nef mutants to induce cytokine/chemokine. In fact, the CC-chemokine MIP-1β is just one of the secretory molecules in the supernatant of infected macrophages. Upon HIV-1 infection, macrophages become an active stage for Nef activities and eventually alter the cellular environment of infection sites by releasing a wide range of factors such as MCSF, TNF-α, IL-6, IL-16, MIP-1α, MIP-β, sCD23, sICAM and ferritin. While the presence of MIP-1α, MIP-1β, sCD23 and sICAM appears to favor viral replication and dissemination, dysregulated expression of MCSF, TNF-α, IL-6, IL-16 and ferritin may contribute to HIV-1 pathogenesis in specific cells and tissues. In this chapter, we determined the presence of MCSF, TNF-α, MIP-1α, IL-6, IL-16 and ferritin in infected macrophages and examined whether their production was also regulated by the KEK motif of Nef.

HIV-associated dementia (HAD), caused by abnormal neuroinflammation and subsequent cell death of neuronal cells, is a common neurological disorder in AIDS patients (250, 251). Nef is considered one of the viral proteins that contribute to the pathogenesis of HAD (252, 253). The expression of Nef is abundant in astrocytes, which appears to be linked to neuroinflammtion (252, 254). Nef has been shown to recruit leukocytes into the central nervous system (CNS) (227). Although rNef itself is toxic to neurons, secretion of a large amout of Nef proteins *in vivo* is still questionable. However,

the endogenous expression of Nef in astrocytes has been shown to induce cytokines and chemokines that may be toxic to neurons (230, 255), probably by manipulating cell signaling pathways. This indicates an indirect Nef-mediated killing effect on neurons. In this chapter, some evidence will be presented to show that Nef induces neurotoxins in the supernatants of Nef-expressing macrophage. Furthermore, we also evaluated the impact of mutations in the KEK motif on this Nef-mediated neuronal cell death effect.

The function of Nef to enhance virus infectivity is not fully understood. It is generally accepted that this function requires the presence of Nef in virus-producing cells rather than in the target cells (113-115). Importantly, it has been shown that co-expressing Nef *in trans* can restore the impaired infectivity of Nef-deficient virus (113, 115), but the essential compensation made by Nef for the intrinsic property of infectious virions remains unclear. Recently, a cellular factor dynamin2 (Dyn2) has been identified as the binding partner of Nef that is required for enhancement of virus infectivity, but the mechanism has not been elucidated (134). In this chapter, we examined the ability of the K^{92}EK/3A mutant to enhance virus infectivity as well as its association with cellular Dyn2 by co-immunoprecipitation.

Result

The KEK motif is dispensable for down-regulation of CD4 and MHC-I

Down-regulation of cell surface CD4 and MHC-I molecules is one of the most studied activities of Nef. Domains within HIV-1 NL4.3 Nef such as $L^{164}L$, $E^{62}EEE$ that independently regulate CD4 and MHC-I down-modulation are not required for MIP-1β induction (Fig. 3-1). We next examined whether mutagenesis of the $K^{92}EK$ motif impaired the ability of Nef to promote down-regulation of CD4 and MHC-I molecules. To this end, Jurkat Tag cells that have both cell surface CD4 and MHC-I were co-transfected with plasmids expressing wild-type or mutant Nef proteins as well as a GFP expression vector (Fig. 4-1). We compared the activity of the $K^{92}EK/3A$ mutant to a $W^{57}L/AA$ Nef mutant that has been previously shown to inactivate Nef-dependent CD4 down-regulation as well as an $E^{62}EEE/4A$ mutant that abrogates Nef-dependent MHC- I down-regulation (83, 93). As expected, the $W^{57}L/AA$ Nef mutant was specifically impaired for CD4 down-regulation yet retained the ability to down-regulate MHC- I and conversely, the $E^{62}EEE/4A$ Nef mutant retained the ability to down-regulated MHC-II but lacked the ability to down-regulate CD4. Compared to mock controls, wild-type Nef significantly down-regulated both CD4 and MHC-I expression on cell surface, and more importantly the $K^{92}EK/3A$ mutant similarly down-regulated both CD4 and MHC-I as well. Therefore, the KEK motif is dispensable for CD4 and MHC-I down-regulation by Nef.

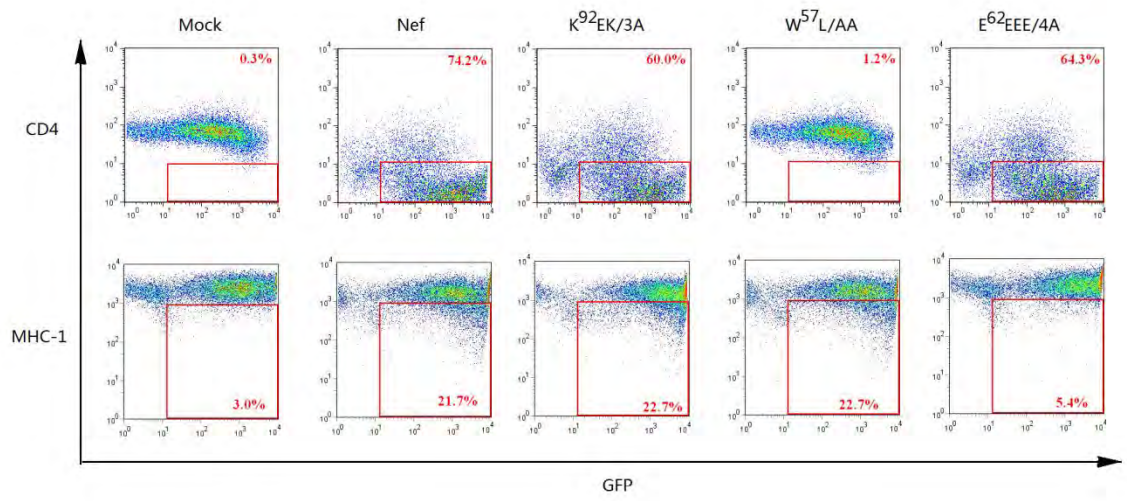

Figure 4-1: The $K^{92}EK$ motif in HIV-1 Nef is dispensable for CD4 and MHC-I down-regulation. Jurkat Tag cells were co-transfected with SRα plasmids expressing the indicated Nef alleles and a GFP expression vector at a molar ratio of 3:1. After 24 h, transfected cells were stained for cell surface CD4 or MHC-I and analyzed by FACS.

Release of other factors by macrophages, in addition to MIP-1β, also requires the KEK motif of Nef

We observed the Nef-dependent MIP-1β induction in infected macrophages and identified the KEK motif as the determinant of Nef-dependent MIP-1β induction. However, previous studies have demonstrated that a variety of factors, in addition to MIP-1β, are also induced in infected macrophages and DCs, for example, MCSF, TNF-α, MIP-1α, IL-6, IL-16 and ferritin (1, 178, 198) (Swingler, unpublished data). We examined the presence of these molecules in the supernatants of infected macrophage cultures and determined the correlation of their induction with Nef expression and the KEK motif. To this end, we infected macrophages with viruses expressing no Nef (ΔNef), wild-type Nef (Nef) or the K^{92}EK/3A mutant (K^{92}EK/3A) and measured the expression level of each factor by ELISA (Fig. 4-2). As a reference, production of MIP-1β was Nef-dependent and was significantly reduced in the supernatants of macrophages infected by the K^{92}EK/3A virus. The same pattern was observed for MIP-1α production. We also detected the presence of IL-6 and IL-16, but at a much lower level compared to MIP-1α and MIP-1β. However, their induction by wild-type Nef virus was still higher than that by ΔNef or KEK^{92}/3A mutant virus. In contrast, a large amount of MCSF production was induced independently of Nef expression or the KEK motif, which is consistent with our previous data showing that MCSF induction required the viral envelope glycoprotein and was Nef-independent (256, 257). Although TNF-α induction by Nef was reported by other groups (197, 198),

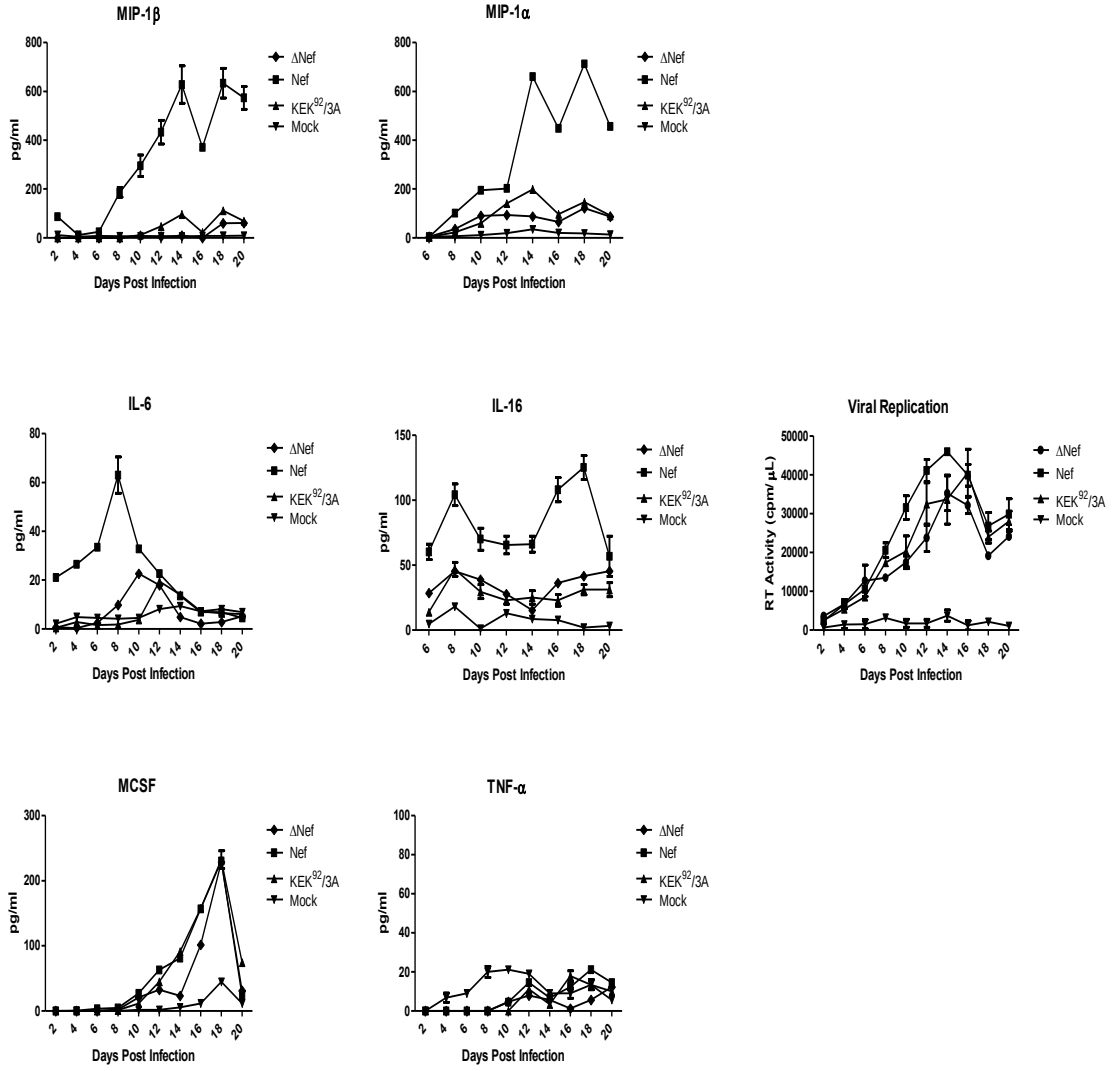

Figure 4-2: Advanced analysis of chemokine and cytokine production induced by the K^{92}EK/3A mutant. Macrophages were infected with HIV-1 viruses with no Nef, wild-type Nef and K^{92}EK/3A mutant. The production level of each factor was determined by ELISA and viral replication was gauged from levels of reverse transcriptase activity in supernatants of macrophage cultures.

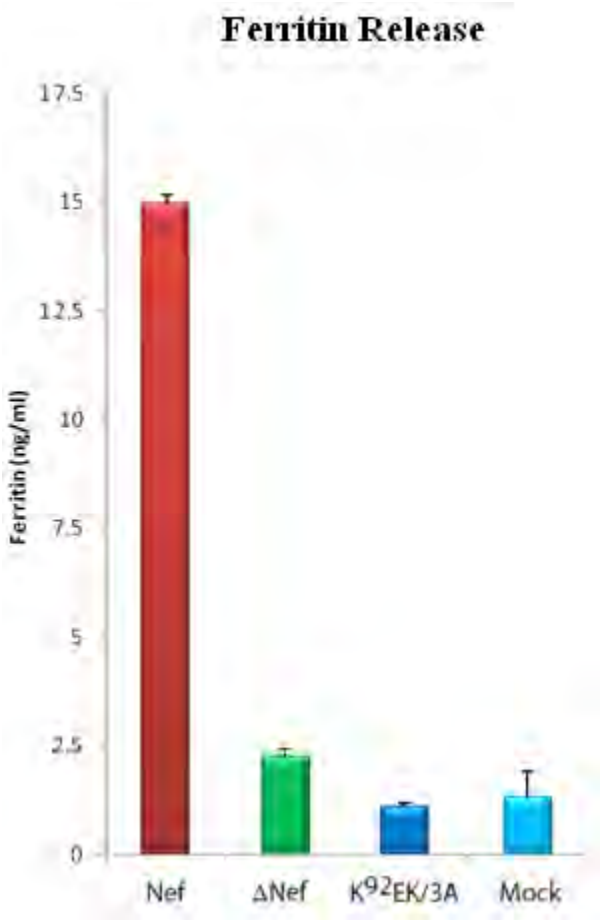

Figure 4-3: The KEK motif is required for Nef-dependent ferritin release from macrophages. Ferritin concentrations were measured by ELISA in the supernatants of macrophages expressing wild-type HIV-1 Nef, no Nef or KEK mutant and mock-infected macrophages (Swingler, unpublished data) .

we only observed a background level of TNF-α production and no correlation with the Nef expression or the KEK motif. Furthermore, we examined the ferritin realease in macrophage supernatants and found that macrophages expressing Nef but not the K^{92}EK/3A mutant produced elevated levels of ferritin relative to ΔNef virus or mock infected macrophages (Fig. 4-3).

Supernatants of macrophages infected with viruses expressing wild-type Nef but not the K^{92}EK/3A mutant induce neuronal apoptosis

HIV-1 infection of macrophages leads to the release of soluble factors in supernatants that may have neurotoxic activities (258, 259). To determine whether Nef induces secretion of neurotoxins from infected macrophages, we initiated spreading infection in macrophages with ADA or ADA-ΔNef viruses, and mock-infected cells as control. We harvested supernatants from infected macrophages and monitored RT activity and MIP-1β production. NT2.N neuronal cells were incubated with the conditioned macrophage supernatants for 48 hours then neuronal apoptosis was evaluated by the Caspase-Glo 3/7 assay. As expected, both ADA and ADA-ΔNef viruses were able to initiate spreading infection in macrophages (Fig. 4-4, upper panel), but MIP-1β induction was only evident in supernatants from ADA but not ADA-ΔNef infected macrophages (Fig. 4-4, upper panel), which was also an indicator of Nef-dependent production of secretory factors in the supernatants. Importantly, neuronal cells displayed more severe cell death when

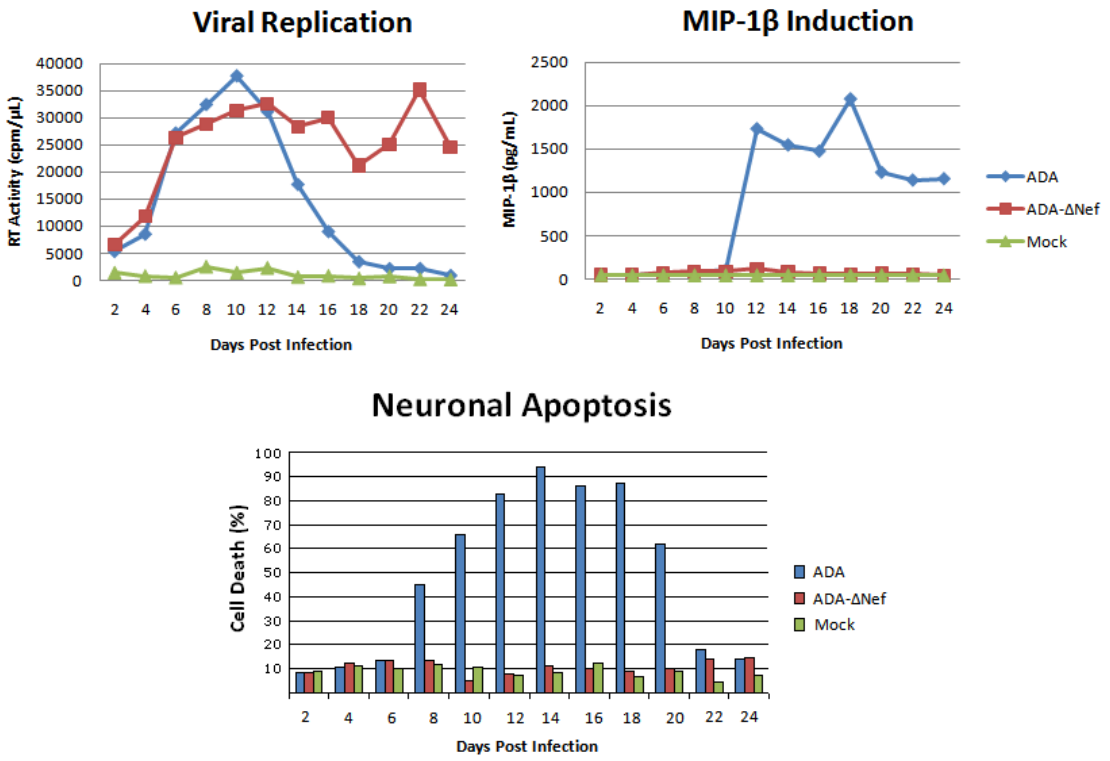

Figure 4-4: Induction of neurotoxicity in macrophage supernatants by Nef. Macrophages were infected with ADA, ADA-ΔNef or mock-infected. Supernatants were collected at indicated time points. Upper panel, RT activity and MIP-1β production in the supernatants were measured to monitor viral replication and release of secretory factors, respectively. Lower panel, the collected macrophage supernatants were used to treat neuronal cells for 48 hours and induction of cell death was determined by Caspase-Glo 3/7 assay (Lower panel: Stranska, unpublished data).

incubated with conditioned supernatants from ADA infected macrophages than those treated with supernatants from ADA-ΔNef or mock-infected macrophages (Fig. 4-4, lower panel). Interestingly, neuronal cell death caused by the supernatants derived from ADA- infected macrophages appeared to correlate with MIP-1β levels in the supernatants. Nevertheless, immunodepletion of MIP-1β did not inhibit neurotoxic activity of the supernatants (Stranska, unpublished data), indicating that an unknown neurotoxin was induced by Nef. We then examined whether mutations in the KEK motif impaired Nef-mediated neurotoxicity (Fig. 4-5). The results showed that the K^{92}EK/3A mutation significantly reduced neuronal apoptosis induced by the conditioned macrophage supernatant by 64% compared to wild-type Nef ($P<0.001$). Therefore, the KEK motif is also a major contributing factor in Nef-induced neurotoxicity.

The K^{92}EK/3A mutation has no significant effect on virus infectivity in macrophages and PBLs.

The molecular basis for infectivity enhancement by Nef remains unclear and is also complicated by the fact that viruses harboring Nef mutations in distinct functional motifs may present partial or full infectivity loss. We have demonstrated that the K^{92}EK/3A mutant was defective in chemokine induction yet was still able to down-regulate both CD4 and MHC-I, indicating that mutagenesis of the KEK motif did not result in global destruction of Nef functions. We next examined how mutations in the KEK motif affect virus infectivity in macrophages and PBLs by taking advantage of an HIV-1 reporter proviral clone NL4-3.Luc.R-E-. This clone is Nef-deficient with a luciferase gene in the

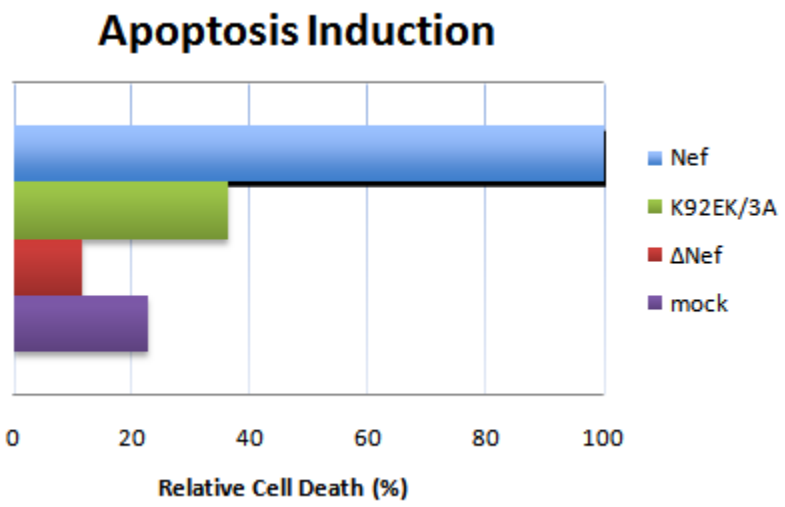

Figure 4-5: Reduced induction of neurotoxicity in macrophage supernatants by the K^{92}EK/3A mutant. Neuronal cells were cultured with conditioned supernatants from macrophages infected with viruses harboring Nef and Nef mutants or mock-infected macrophages. 48 hour later, cell death in neuronal cells was determined by Caspase-Glo 3/7 assay. (Stranska, unpublished data)

position of Nef. The envelope gene is also mutated with a frameshift, and thus expression of X4- or R5- envelope proteins must be provided *in trans*. Therefore, luciferase expression indicates a single-round infection. By co-expressing wild-type Nef or the K^{92}EK/3A mutant *in trans*, we were able to evaluate their influence on virus infectivity in macrophages and PBLs.

To infect macrophages, reporter viruses were complemented with M-tropic envelope, as well as wild-type Nef or the K^{92}EK/3A mutant, with or without VSV-G pseudotyping, and luciferase activity was measured 48 hour post infection. Based on the levels of luciferase expression, macrophage infection initiated by all three non-pseudotyped viruses was at a similar low level and no enhancement of virus infectivity promoted by wild-type Nef or the K^{92}EK/3A mutant was observed ($P>0.05$) (Fig.4-6, left panel). The same viruses, when tested in TZM-bl cells, were highly infectious. However, the infectivities of the viruses complemented with or without wild-type Nef were not statistically different ($P>0.05$) and the K^{92}EK/3A mutation exhibited no significant effect on virus infectivity compared with wild-type Nef and no Nef expression ($P>0.05$) (Fig. 4-6, right panel). Therefore, we conclude that K^{92}EK/3A mutant has no significant effect on virus infectivity in macrophages.

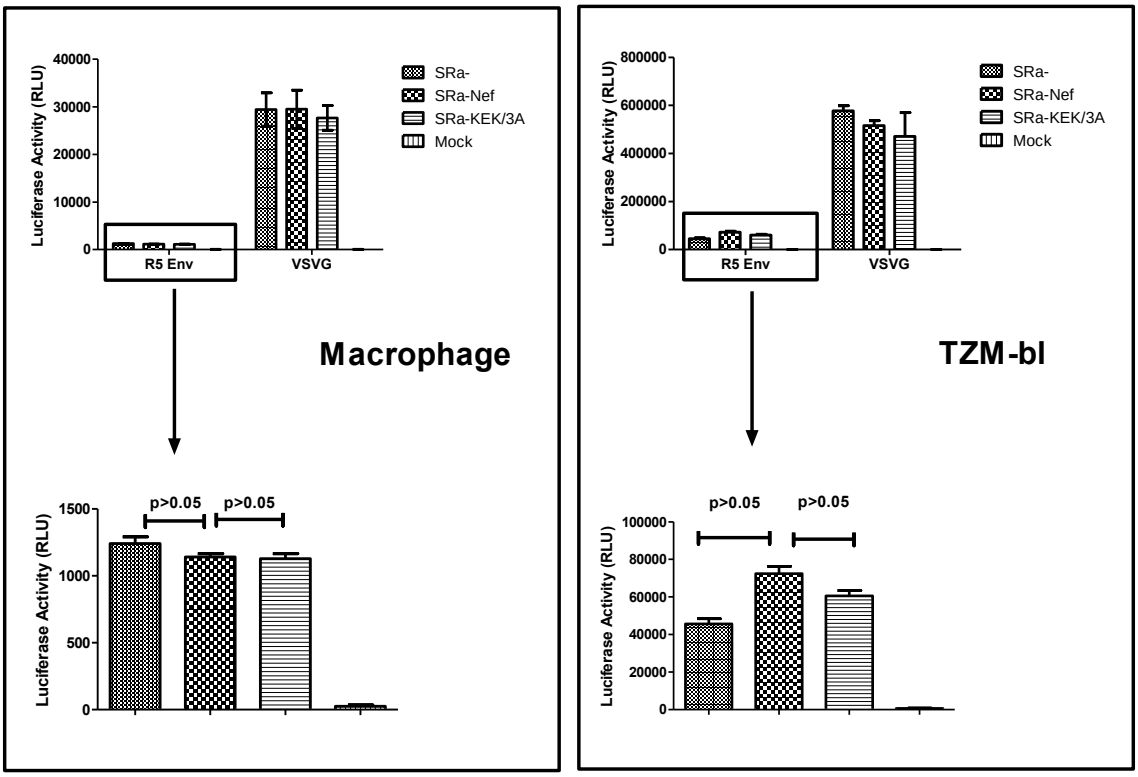

Figure 4-6: Infectivity analysis of ADA-ΔNef complemented with *in trans* expression of Nef or the K^{92}EK/3A mutant in macrophages. Reporter viruses NL4-3.Luc.R-E- were complemented with M-tropic envelope and wild-type Nef or KEK mutant, with or without pseudotyping with VSV-G. Same amount of viruses normalized by RT were used to inoculate macrophages (left panel) and TZM-bl cells (right panel) for 2 hour and virus infectivity was measured 48 hour post-infection. The results are presented as luciferase activity quantified by the bright-glo assay system.

We then conducted a similar experiment by complementing reporter viruses with T-tropic envelope and examined virus infectivity in PBLs by measuring luciferase signals. Similar to the results from the infecticity assay in macrophages, T-tropic viruses complementing with no Nef, wild-type Nef and the K^{92}EK/3A mutant exhibited no significant difference in virus infectivity in PBLs ($P>0.05$) (Fig. 4-7, left panel). The virus infectivities, when tested in Ghost cells, were also statistically indistinguishable ($P>0.05$) and the K^{92}EK/3A mutation had no significant effect on virus infectivity compared with wild-type Nef ($P>0.05$) (Fig. 4-7, right panel). In addition, all three viruses showed indistinguishable infectivities independently of Nef expression when they were pseudotyped with VSV-G ($P>0.05$) (Fig. 4-6, 4-7).

Collectively, we conclude that the K^{92}EK/3A mutation has no significant effect on virus infectivity in macrophages and PBLs.

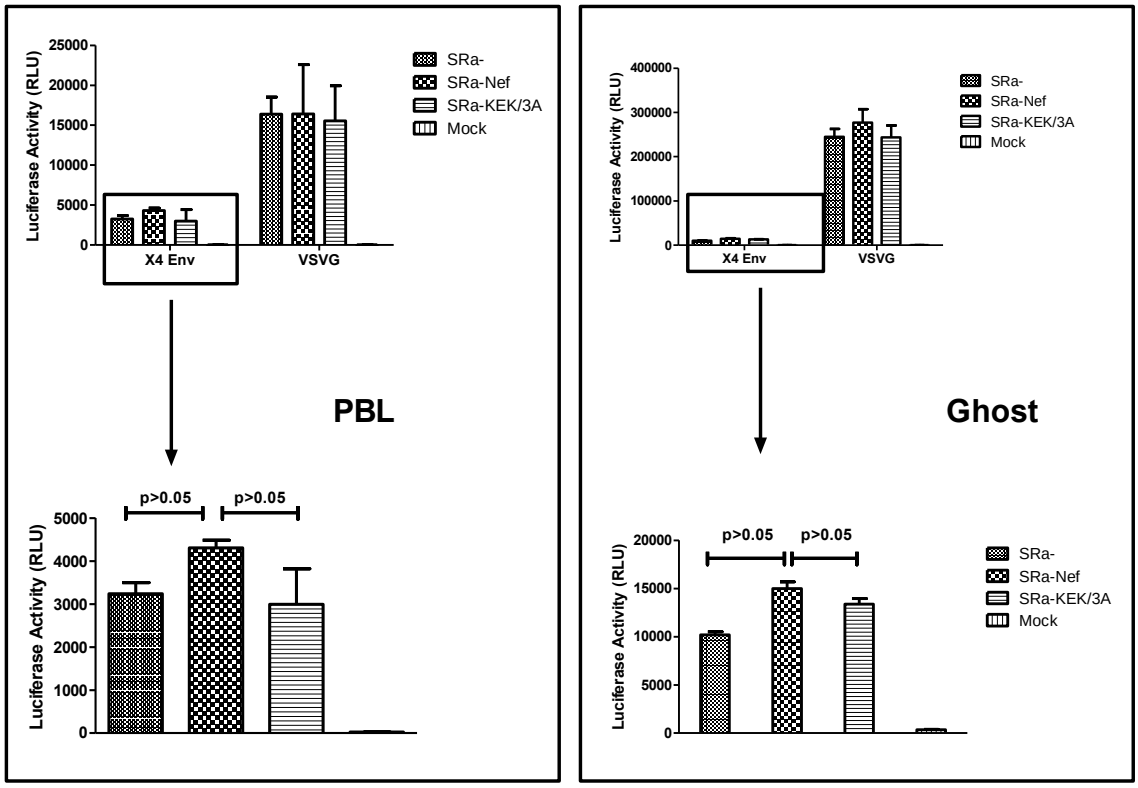

Figure 4-7: Infectivity analysis of ADA-ΔNef complemented with *in trans* expression of Nef or K^{92}EK/3A mutant in PBLs. Reporter viruses NL4-3.Luc.R-E- were complemented with T-tropic envelope and wild-type Nef or KEK mutant, with or without pseudotyping with VSV-G. Same amount of viruses normalized by RT were used to inoculate PBLs and Ghost cells and virus infectivity was measured on day 6 and day 2 post infection, respectively. The results are presented as luciferase activity quantified by the bright-glo assay system.

Discussion

In this chapter, we presented the results of a comprehensive functional assessment of the HIV-1 Nef K^{92}EK/3A mutant defective in MIP-1β induction.

The K^{92}EK/3A mutant remained highly active for CD4 and MHC-I modulation, the two classical functions of Nef. This fully uncoupled the molecular basis for Nef-induced cell surface molecule down-regulation from that of Nef-regulated chemokine induction. More importantly, it suggests that the defect of the K^{92}EK/3A mutant in chemokine induction was not due to a destructive effect on global Nef functions.

The Jurkat Tag cell line made it possible to test both CD4 and MHC-I down-regulation by Nef in one cell culture system. The cells had abundant CD4 and even higher MHC-I expression on the cell surface that were efficiently down-regulated upon Nef expression. Although we gated on the GFP positive population that represented the transfected cells to monitor the down-regulation effect, it should be noted that this down-regulation effect also appeared evident in GFP negative cells as well. This is due to the molar ratio of the co-transfected plasmids and the high efficiency of the nucleofection method. Since we co-transfected three fold more Nef or Nef mutant-expressing vectors than the GFP-expressing vector and since more than 70% cells were GFP positive, it was not suprising that almost all of the transfected cells were expressing Nef.

Although mutagenesis of the KEK motif did not affect Nef-induced CD4 and MHC-I down-regulation, it impaired the induction of several other secretory factors such as MIP-

1 α, IL-6, IL-16, ferritin as well as possible unknown neurotoxins in macrophage supernatants. The spontaneous Nef-dependent induction of MIP-1α and MIP-1β is consistent with the previous data from the macrophage infection experiments (1). In different donors, the induction patterns of MIP-1α and MIP-1β appeared to be the same, which was characterized by two concentration curves that displayed very similar trends during macrophage infection. The MIP-1α and MIP-1β curves seemed to correlate with the RT curves and reached their peaks at the same time. Indeed, the induction of MIP-1α and MIP-1β was dependent on viral replication especially during spreading infection in macrophages. One possible explanation would be that Nef expression had to be accumulated to a threshold to trigger chemokine induction. In agreement with this, the intracellular concentration of Nef in T cells has been described to dictate whether CD4 and MHC-I molecules are down-regulated or T cell activation is affected (260). Although Nef is one of the viral proteins that are expressed at early stages of infection, its expression level can be also limited by infection efficiency. In macrophage infection experiments where virus replicated in a spreading manner, the number of initially infected macrophages was so limited that Nef expression was undectable by western blotting analysis in macrophgaes lysates for the first several days (Dai, unpublished data). With the viral replication proceeding, Nef expression was accumulated in more and more infected macrophages and finally reached a possible threshold for MIP-1α and MIP-1β induction. Furthermore, mutagenesis of the $K^{92}EK$ motif significantly reduced MIP-1α and MIP-1β induction to a similar extent. Therefore, Nef may need the KEK motif for the same pathway to regulate both MIP-1α and MIP-1β production.

In addition to MIP-1α and MIP-1β, we examined the release of other factors during HIV-1 infection in macrophages. Our data suggested that the induction of IL-6, IL-16 and ferritin from macrophage supernatant was also regulated by Nef, which is consistent with studies demonstrating the increased induction of these factors by Nef in a variety of cell types including monocyte, DCs, macrophages, astrocytes and microglial cells (197, 198, 230, 261). Importantly, we found that regulation of IL-6, IL-16 and ferritin release also required the KEK motif. The expanded list of the KEK motif-dependent molecules may suggest a specific role for this motif in switching on the dysregulated release of a broader panel of regulatory factors, which may contribute to HIV-1 replication and pathogenesis.

Unlike MIP-1α and MIP-1β, IL-6 and IL-16 exhibited totally different production curves. Specifically, induction of IL-6 arose at the early stage of the infection prior to the RT peak. In contrast, induction of IL-16 appeared to drop between two peaks at both the early and the late stage of the infection. Other studies confirm that IL-6 is induced early after HIV monocytic infection (262, 263). Since IL-6 is potent activator of C/EBP, it can activate other cytokine genes such as IL-1β (264) and TNF-α (265). Although we did not detect potent IL-1β or TNF-α induction in our experiments probably due to the low level of IL-6 induction itself, this still raises the possibility that early cytokines/chemokine induced by Nef may have regulatory effect on late induced cytokines/chemokine. If this is true, Nef may regulate complicated cytokine/chemokine networks in infected macrophages, although it is still unclear whether Nef requires the KEK motif to activate single or multiple pathways to induce the production of these molecules.

In our macrophage infection experiment, we found a much lower level of IL-6 induction and did not consistently observe Nef-dependent production of IL-1β and TNF-α as reported by another study in which macrophages were treated with recombinant Nef (197). The discrepancy may be a reflection of different mechanisms by which endogenously expressed Nef and extracellular rNef activate signaling pathways in macrophages. In the rNef system, macrophages are exposed to levels of Nef that are artificial and not achievable *in vivo*. Even if internalization of rNef occurs, the results may not represent physiological events induced by intracellular Nef in infected cells. Indeed, two motifs $E^{156}E$ and $D^{174}D$ have been reported to be required for extracellular recombinant Nef to induce MIP-1α and MIP-1β. However, in our macrophage infection experiment, these two motifs appeared to be totally dispensable for MIP-1α and MIP-1β induction in the context of viral infection (Fig. 4-8).

We have demonstrated that Nef induced a wide range of factors in infected macrophage supernatants. In addition, we showed the exsistence of neurotoxic activity in the conditioned media from infected macrophage cultures. Neurotoxin induction appeared to be dependent on Nef and an intact KEK motif. Recently, it was reported that supernatants from Nef-expressing astocytes induced neuronal cell death which was linked to increased IP-10 expression (255). Unfortunately, IP-10 expression was not detectable in the supernatants of our macrophage experiments (Dai, unpublished data). Further studies are needed to reveal the exact identity of the neurotoxic factor(s) as well as the molecular mechanism by which they are regulated by the KEK motif of Nef.

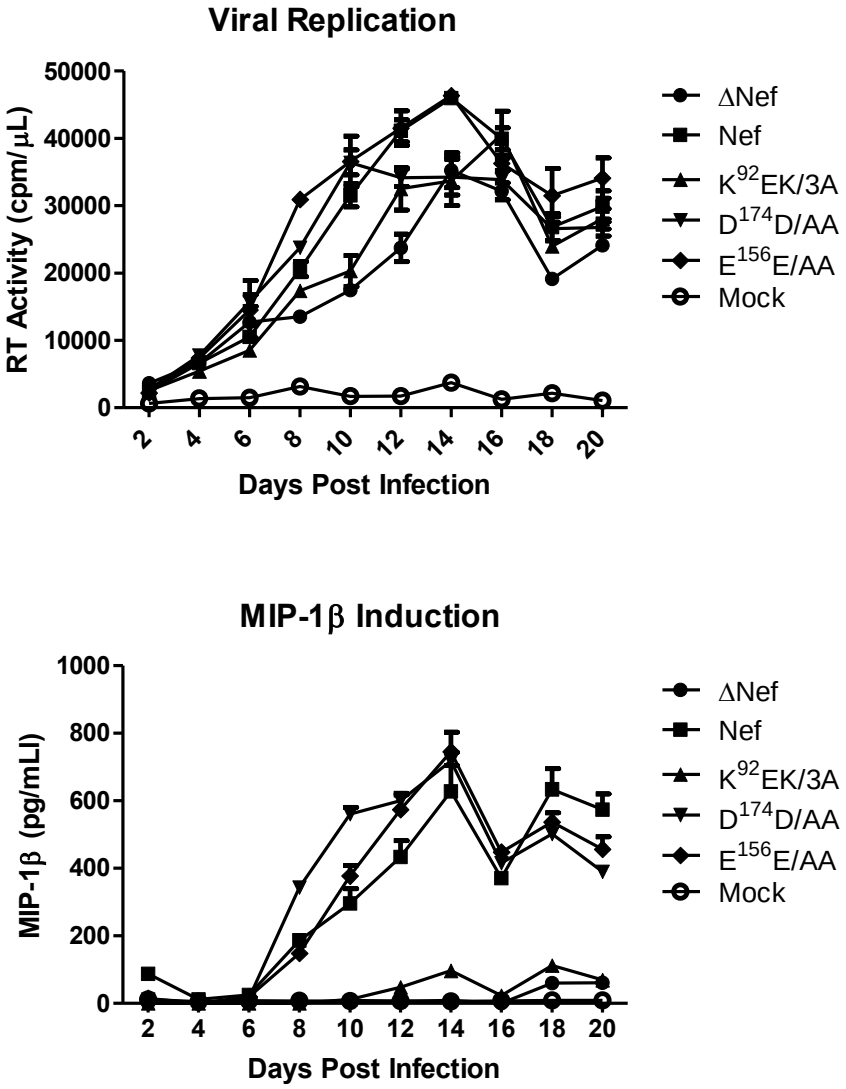

Figure 4-8: The $D^{174}D$, $E^{156}E$ motifs of HIV-1 Nef are dispensable for Nef-induced MIP-1β production in the context of macrophage infection. Macrophages were infected with viruses harboring wild-type Nef and Nef variants containing amino acid substitutions as indicated. Viral replication was determined by RT (upper panel) and MIP-1β production by ELISA (lower panel).

Nef is known to moderately enhance virus infectivity. However, we did not observe evident enhancement of virus infectivity by Nef in either macrophages or PBLs with our infectivity assay system. Furthermore, the K^{92}EK/3A mutation exhibited no significant effect on virus infectivity. Therefore, the KEK motif does not seem to be the determinant of Nef for virus infectivity enhancement. In support of this, both wild-type Nef and the KEK mutant bind to Dyn2 (Fig. 4-9), which is required for Nef-dependent infectivity enhancement (134). A possible reason that they did not exhibit a significant boost effect on virus infectivity in our system is that the experimental conditions might not be optimal, since it has been shown that the boost effect on virus infectivity by Nef may vary depending on virus isolates and producer cell types (116, 266). SF2 Nef exhibited a more significant boost effect on virus infecticvity than NL4.3 Nef (116) and Jurkat T cells have been used as producer cells to observe significant infectivity enhancement (134). These can be utilized in future to optimize our infectivity assay system.

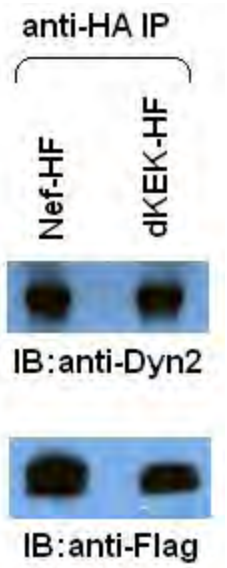

Figure 4-9: Both Nef and KEK mutant bind Dynamin2. 293T cells were transfected with SRα vectors expressing wild-type Nef and Nef mutant lacking the KEK motif that were double-tagged with HA and Flag on C-terminus. Anti-HA immunoprecipitates (IP) prepared from 293T cells lysates were analyzed by immunoblotting (IB) with anti-Dyn2 and anti-Flag.

CHAPTER V.

FINAL CONCLUSION AND PERSPECTIVE

1. Summary

The work presented in this thesis described the identification of a novel functional KEK motif within HIV-1 and SIV Nef proteins that is required for the induction of MIP-1β production from infected macrophages. A series of Nef truncation mutants and various point-mutants were examined to establish that the conserved KEK domain of HIV-1Nef was responsible for MIP-1β production. Mutagenesis in the KEK motif also reduced the Nef-dependent induction of MIP-1α, IL-6, IL-16 and ferritin, as well as the release of neurotoxin in the supernatants of infected macrophages. However, mutation of the KEK motif did not impaired other general functions of Nef in terms of CD4 and MHC-I down-modulation. In addition, mutation of the KEK motif had no significant effect on virus infectivity in macrophages and PBLs. Collectively, this thesis research revealed a specific determinant within Nef that might be critical for a central feature of HIV-1 replication and pathogenicity.

2. General discussions

2.1. Involvement of Env in Nef-dependent MIP-1β induction

In our macrophage infection experiments, we observed the reduced production of several factors in the supernatants of macrophages infected with the ΔNef or K92EK/3A mutant virus. However, it should be noted that in some donors they appeared to be slightly higher than the background of mock infection (Fig. 3-4, 3-5, 4-2, 4-8), which might indicate the involvement of other factors in cytokine/chemokine regulation during HIV-1 infection.

As discussed in chapter IV, we interpreted the correlation of MIP-1α and MIP-1β with viral replication as the accumulation of Nef expression. However, with more viral particles produced during infection, the accumulaton of viral envelope proteins should also be taken into consideration. In fact, studies have demonstrated the ability of recombinant HIV-1 gp120 to induce cytokines and chemokines (267). Thus, it is possible that Nef causes an indirect effect on cytokine/chemokine induction through Env. This model was attractive since it might also help to explain the mechanism of Nef-dependent infectivity enhancement.

We tested this hypothesis by infecting macrophages with wild-type virus (WT), Nef-deficient virus (ΔNef), Env-deficient virus (ΔEnv) and dual deficient virus (ΔNefΔEnv) that were pseudotyped with VSV-G (Fig. 5-1). As expected, ΔNef induced significantly less chemokine induction compared to WT. In contrast, MIP-1β production induced by

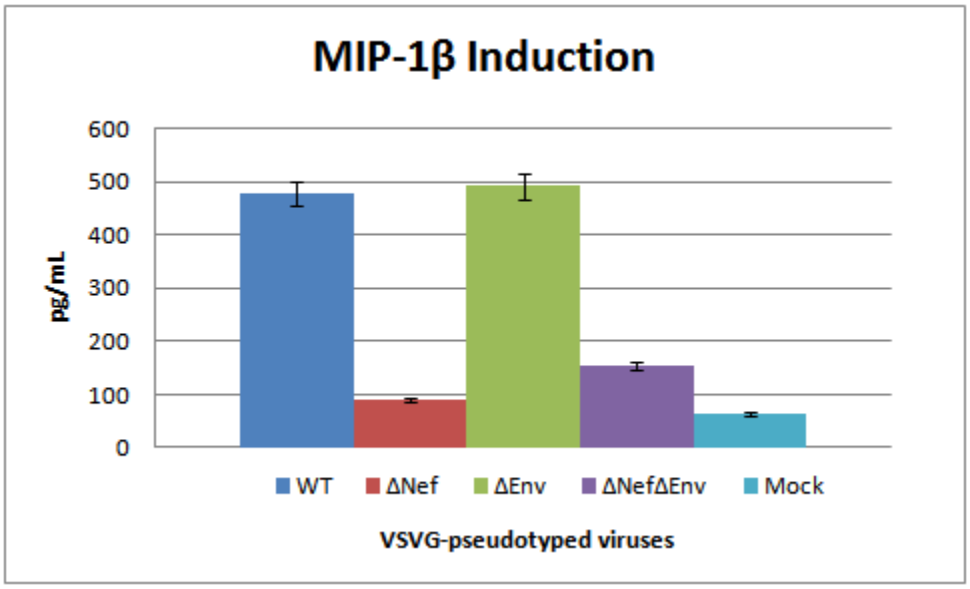

Figure 5-1: Involvement of Env in Nef-dependent MIP-1β chemokine induction. Macrophages were infected with with wild-type virus (WT), Nef-deficient virus (ΔNef), Env-deficient virus (ΔEnv) and dual deficient virus (ΔNefΔEnv) and all viruese were pseudotyped with VSV-G. MIP-1β production in the supernatants was measured by ELISA.

ΔEnv was indistinguishable from that induced by WT. This excluded Env as a major determinant for MIP-1β induction. In addition, there was no synergistic reduction in MIP-1β production induced by ΔNefΔEnv compared to that induced by ΔNef (Fig. 5-1). Furthermore, we used anit-CD4 antibody and CCR5 antagonist TAK779 to block interaction between envelope and receptor and co-receptor, respectiviely, and neither seemed to affect Nef-dependent MIP-1β induction (Fig. 5-2). Collectively, these results did not seem to support the involvement of envelope proteins in Nef-induced MIP-1β chemokine production. However, we did not completely exclude involvement of other viral proteins such as Tat, Vpr that might also have the ability to induce cytokines and chemokines. Therefore, further studies are needed to determine whether Nef works by itself or with other viral factors to regulate cytokine/chemokine production.

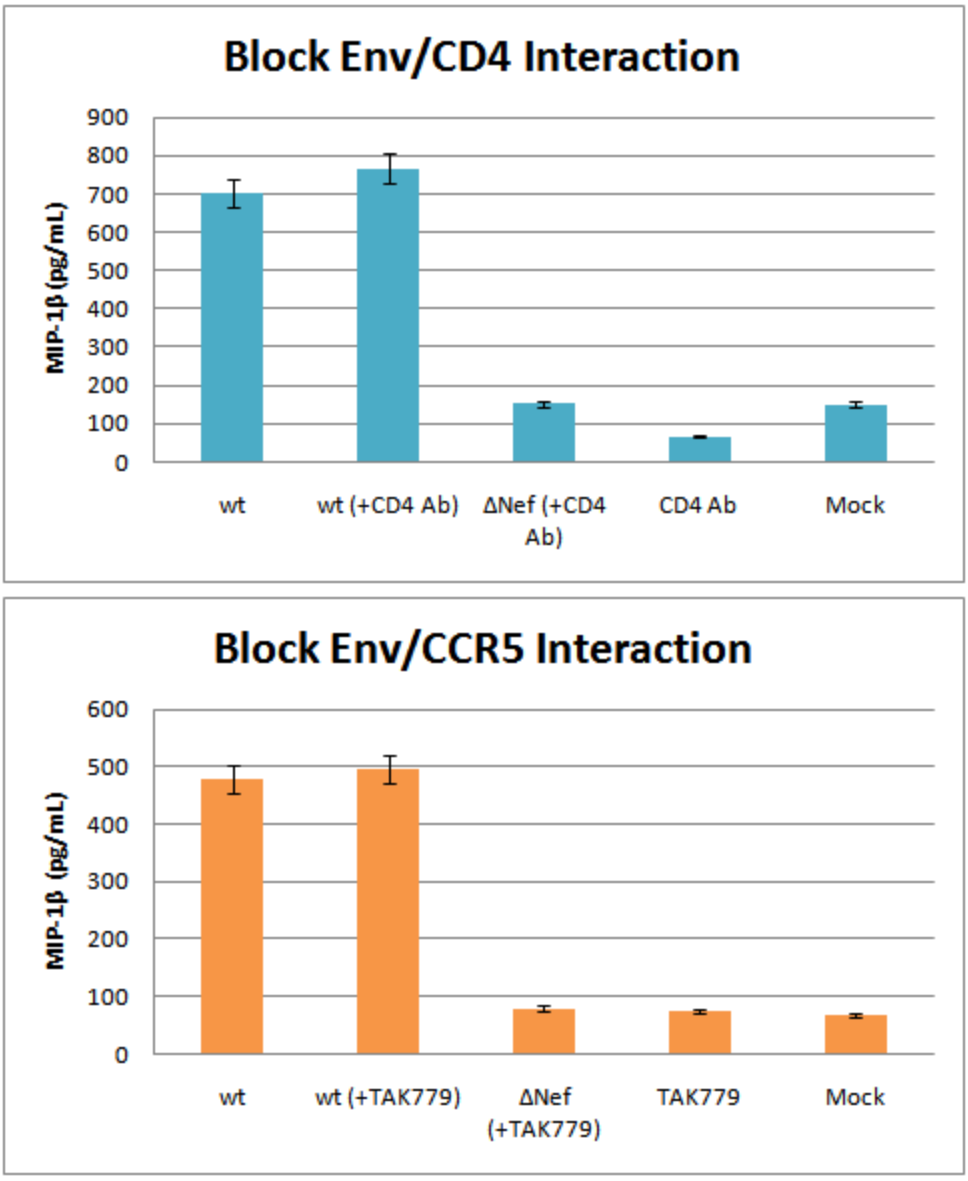

Figure 5-2: Nef-induced chemokine production is independent of gp120 interactions with CD4 and CCR5. Macrophages were infected with VSV-G-pseudotyped wild-type virus (wt) and Nef-deficient virus (ΔNef) in the presence of an anti-CD4 antibody (upper panel) or a CCR5 antagonist TAK779 (lower panel).

2.2. Nef and CD40 signaling pathways

While studies have provided a substantial amount of observations regarding Nef-induced cytokine/chemokine in macrophages, understanding of the signaling pathways that are involved is still very limited. CD40 is an important cell surface marker on macrophages and CD40-CD40L interaction is thought to play a physiological role in immune regulation of macrophages. CD40-induced activation of macrophages results in morphological changes and cytokine/chemokine secretions (231, 268). Although CD40 itself does not harbor kinase acitivity, stimulating CD40 with CD40L activates a complex network of signal transduction pathways that involve various kinases and TNF-R associated factors (TRAFs). Activation of NF-κB has been described as one major downstream effect of CD40-induced pathways (269). In the quiescent state, NF-κB is predominantly present in the cytoplasm in association with its inhibitors IκB -α and IκB-β. CD40-CD40L interaction triggers signals that lead to NF-κB activation by releasing NF-κB from IκB inhibition. Once NF-κB gets into nucleus as a transcriptional complex, it can recognize promoter sequences of cytokine/chemokine genes including IL-1, IL-6, IL-8, IL-10, IL12, TNF-α, MIP-1α, and MIP-1β and activate their expression (270-273). Therefore, it is possible that Nef may mimick or intersect the CD40 signaling pathways to alter cellular conditions such as NF-κB activation in infected macrophages. In support of this, endogenous expression of Nef is sufficient to functionally substitute for CD40L stimulation to induce the production of sCD23, sICAM and ferritin by macrophages in a NF-κB dependent manner (178, 231). In addition, extracellular rNef induces the release

of IL-1, IL-6, TNF-α, MIP-1α, and MIP-1β by macrophages, which is also correlated with NF-κB activation (197).

To futher examine the relationship between Nef and CD40 signalling, we infected macrophages with VSV-G-pseudotyped HIV-1 viruses expressing no Nef (ΔNef), wild-type Nef (Nef) or K^{92}EK/3A mutant (K92EK/3A), and afterwards stimulated infected macrophages with CD40L (Fig. 5-3). MIP-1β induction was measured after viral infection as well as after CD40L stimulation. The rationale is that, if Nef occupies the only machinery in the CD40 signaling pathway that triggers MIP-1β induction, one should expect that CD40L simulation would induce less MIP-1β production by macrophages that have been infected with Nef-expressing viruses, because overexpression of Nef may have saturated the signaling pathway and weakened the subsequent MIP-1β induction by CD40L. However, expression of Nef did not seem to interfere with CD40L-induced MIP-1β production. The net amount of CD40L-induced MIP-1β production was close in all infected macrophages regardless of Nef expression. Thus, it is possible that CD40L stimulation triggers multiple parallel pathways that lead to NF-κB activation and Nef might only intersect one or several specific pathways.

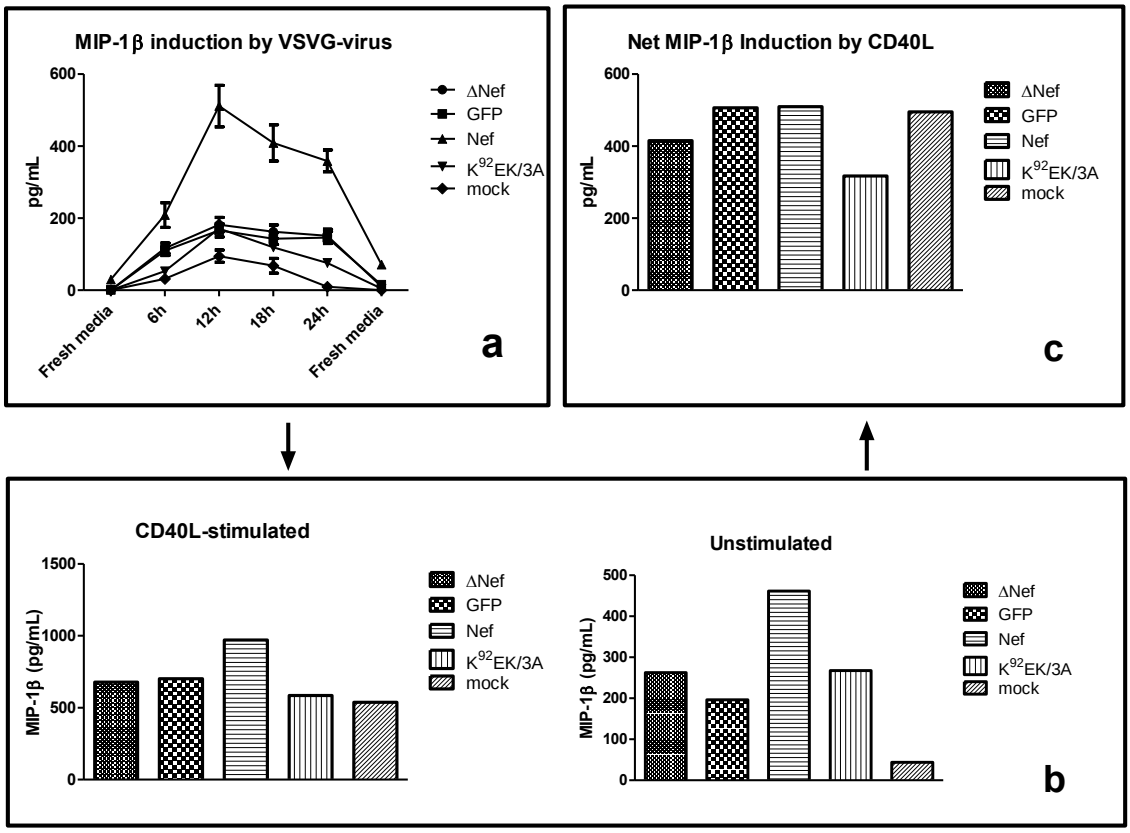

Figure 5-3: CD40L-induced MIP-1β production is not affected by Nef expression from previous viral infection. a) Macrophages were infected with VSV-G-pseudotyped viruses as indicated. Infected cells were washed and cultured in fresh media. For the next 24 h, MIP-1β production in supernatants was monitored at indicated time points. Afterwards, cells were washed again and fresh media was added. b) Infected macrophages were cultured in fresh media with (CD40L-stimulated) or without (Unstimulated) CD40L stimulation. After 24 h, MIP-1β production in supernatants was measured. c) Net MIP-1β production by CD40L stimulation was caculated by subtracting the MIP-1β background in unstimulated group from the gross MIP-1β production in CD40L-stimulated group.

Indeed, it has been described that several events in CD40 signaling pathways might be involved in NF-κB activation. For example, activation of phosphoinositide 3 kinase (PI3K) is one of the early kinase events after CD40 activation. PI3K can activate IκB kinase (IKK) that phosphorylates IκB to mediate NF-κB activation. CD40 activation is also coupled with activation of the ERK signaling pathway (274). PI3K and ERK signaling pathways are both important for NF-κB activation in monocytic cells (275). In addition, regions of CD40 which interact with different intracellular TRAF members have been identified. Among the TRAF proteins, TRAF2, TRAF5 and TRAF6 are able to activate signaling pathways of NF-κB activation by inducing IKK activation. Thus, the interaction between CD40 and TRAFs adds to more complexity of CD40-induced NF-κB activation. Although some evidence suggests Nef may intersect the CD40 signaling pathway in HIV-1 infected macrophages, it remains to be fully established what stage of the CD40 signaling pathways is hijacked by Nef. To this end, future experiments by genetically or chemically inhibiting certain cell signaling pathways in macrophages might be helpful. In addition, the identification of the KEK motif offers a functionally defective mutant that we can utilize to eventually clarify the specific pathways that are regulated by Nef.

2.3. The role of the KEK motif

Technically, delivery of Nef into macrophages can be provided by extracellular rNef or endogenous Nef expression through viral transduction using Adeno, M-tropic HIV-1 ADA or VSV-G-pseudotyped viral systems (1, 197, 198). Nevertheless, Nef seems to induce similar effects in macrophages no matter how it is delivered. Consistently, Nef-dependent MIP-1β induction has been confirmed in all the systems with a common finding that myristoylation of Nef is not strictly required. Therefore, it is possible that Nef may not have to be targeted to the cell membrane to be functional, and an abundant pool of cytoplasmic Nef proteins may be sufficient to trigger signals for gene activation. It has been shown that extracellular rNef can be efficiently internalized by macrophages and two motifs $D^{173}D$ and $E^{154}E$ are required for the subsequent NF-κB activation (197). In contrast, these motifs seem to be largely dispensable for MIP-1β induction by *de novo* synthesized Nef during HIV-1 infection in macrophages (Fig. 4-8). Since these two motifs seem to be more involved in Nef trafficking via the endocytotic machinery (88, 276), MIP-1β induction by the extracellular rNef may need transportation of internalized rNef to specific intracellular compartments to be functional for subsequent gene activation. Clearly, mutations in the DD, EE have been shown to impair Nef-induced CD4 down-regulation (87, 88). Compared to the DD, EE motifs, the KEK motif should represent a more essential feature of Nef for signal transduction. Since mutagenesis in this motif does not affect general activities of Nef such as CD4 and MHC-I down-regulation, we do not expect mutations in this motif to alter the subcellular distribution of

Nef in macrophages. Instead, the functional losses of the K^{92}EK/3A mutant may reflect a subtle but important event that is regulated by Nef. Remarkably, this motif is highly conserved in Nef alleles from different HIV-1, SIV and HIV-2 clades, which indicates a strong evolutionary pressure for primate lentiviruses to maintain this functional motif. However, futher experiments should be done to determine whether mutations in the KEK motif can be quickly reverted after passage in macrophages or in SIV-infected monkey models.

At present, the exact role of the KEK motif is unclear. We did one experiment to determine the contribution of each residue within the K92EK motif by individually substituting K92, E93 or K94 for alanine and evaluating the impact on chemokine induction in infected macrophage. While K^{92} was largely dispensable for Nef-dependent induction of MIP-1β in infected macrophages, E^{93} and K^{94} were necessary for the induction of MIP-1β by HIV-1 Nef (Fig. 5-4). Therefore, among the residues of K92EK motif, residues E93 and K94 seem to make the most contribution to chemokine induction. Since this charged motif is structurally exposed with full surface accessibility, K^{92}EK may lie in the region of HIV-1 Nef that interfaces with cellular factors. The carboxylate side chains of these residues may protrude and form hydrogen bonds with a potential binding partner. In fact, a similar model has been established in T cells where Nef forms a complex with Pak-2 and others cellular factors that initiate downstream signaling events to regulate cell activation (136, 146). While this may similarly occur in infected macrophages, it seems unlikely that the loss of two residues E93 and K94 could completely destabilize such interactions. Althought we identified the KEK motif by

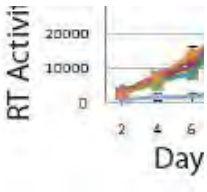

Figure 5-4: Role of individual Nef K^{92}EK residues in MIP-1β induction.

Macrophages were infected with viruses expressing wild-type HIV-1 Nef or Nef mutants harboring individual alanine substitutions within the KEK domain. Left panel, viral replication was determined by RT activity. Right panel, MIP-1β production was measured by ELISA.

selectively examining several conserved candidates within the 31 amino acid region, a complete alanine scanning throughout the region might reveal more information regarding other necessary motifs. This will improve our understanding of the KEK motif possibly as a team member in cellular regulation by Nef.

Moreover, the glutamate and lysine residues in this motif might have some indications. Although Nef has the ability to bind several kinases, so far there is no evidence to support any kinase activity of Nef itself and we have found no obvious similarities for the KEK motif to any known kinase domains. However, it is possible that Nef may interact with a cellular kinase to trigger the signaling pathway. The glutamate residue may be important for such interaction, since glutamate-based motifs have been previously shown to play an important role in Nef's interaction with cellular partners (88, 93, 96). Further mutagenesis analysis with E93/D and E93/Q mutants will address the importance of the side chain and the negative charge of the E93 residue. In addition, the lysine residue is a good position for ubiquitination modification. Indeed, among the 10 lysines residues of Nef, ubiquitination of K92 and K94 has been confirmed (89). Presumably, poly-ubiquitination of Nef may lead to different physiological results depending on the type of poly-ubiquitin chains. Nef may form a complex with an inhibitory factor for a certain signaling pathway and ubiquitination mediated by K48 linkage could be a degradation signal to remove the inhibition. Recently, more focus has switched to the regulatory effect of K63 ubiquitination (277). Some evidence has linked K63 ubiquitination of TRAFs to CD40-induced NF-κB activation (278). If Nef does indeed mimick a CD40 signaling pathway in infected macrophages, it is possible that Nef triggers NF-κB

activation the same way as TRAFs through regulatory ubiquitination. Nevertheless, future experiments should be performed to test these possibilities in order to fully understand the exact role of the KEK motif.

2.4 A proposed model

It has been established in T cells that expression of Nef triggers a transcriptional program that shares 97% similarity to that induced by anti-CD3 T cell stimulation (279). Our studies support a similar idea that Nef manipulates cell signaling pathways in macrophages. The direct result of dysregulated macrophage activation is the release of a wide range of cellular factors; these include not only chemokines/cytokines such as MIP-1α, MIP-1β, IL6, IL-16 but also large molecules such as ferrin and even factors that are neurotoxic. These molecules cause various effects on bystander cells; T cells are recruited and activated to better support viral replication. B cells are hyperactivated and thus dysfunctional. Neuronal cells are exposed to neurotoxins and suffer from apoptosis. All these contribute to a model in which Nef alters cellular conditions of macrophages that promote viral dissemination and lead to pathogenesis. Our studies indicate the KEK motif of Nef as the molecular basis for this model. This paves the way for future studies to explore machanisms behind the model.

3. Future directions

Identification of the KEK motif is just a start of another Nef story in which many blanks are to be filled. What is the exact molecular mechanism of Nef regulation in macrophages? How does the KEK motif fit in this model? Which signaling pathways are involved? Is the KEK motif also important in regulation of other cells such as T cells, B cells, DCs? How and to what extent does the KEK motif affect other Nef functions *in vitro*? How do these effects contribute to the totality of Nef function *in vivo*?

Among all the questions we may have, two seem to be of higher priority. Firstly, the biological significance of the KEK motif should be evaluated *in vivo*. The importance of the KEK motif can be further analyzed in transgenic mouse models and the SIV/macaque model of HIV-1 infection. Such experiments should determine the selective pressure of the KEK motif after passage as well as the impact of the KEK motif on viral fitness and pathogenicity *in vivo.* On the molecular basis, future studies should identify the cellular factors that may be regulated by Nef via the KEK motif. This will be the key to a better understanding of the exact molecular mechanisms. To this end, we have got some interesting preliminary results in our pilot co-immunoprecipitation experiments. We repeatedly observed a ~70kDa band in the co-immunoprecipitates with the wild-type Nef but not with a Nef mutant lacking the KEK motif (Fig. 5-5). Although the identity of this band remains to be confirmed, the existence of such potential factors may guide us to the next page of the KEK story of Nef.

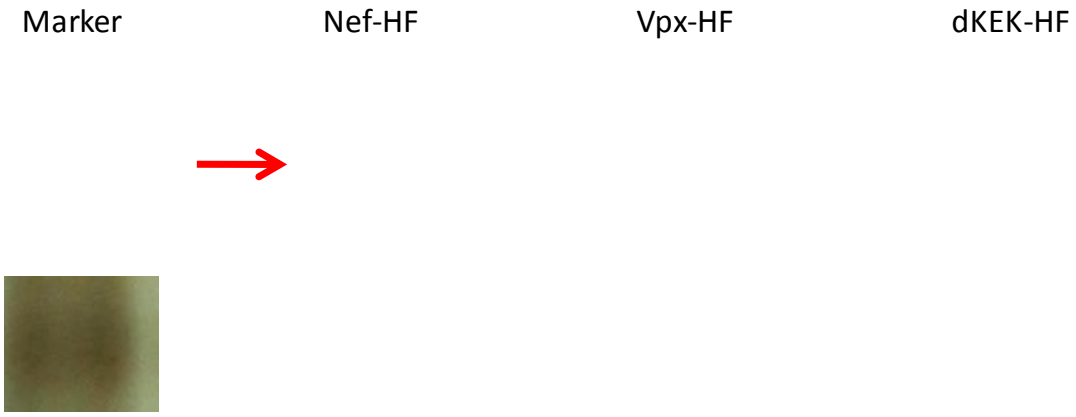

Figure 5-5: A 70-kDa protein co-precipitates with Nef but not with the ΔKEK mutant. Anti-HA immunoprecipitates from 293T cells transiently expressing Nef-HF, dKEK-HF or Vpx-HF were analyzed by SDS/PAGE and silver staining.

CHAPTER VI.

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